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(54) Title: ANTI-BACTERIAL VACCINE COMPOSITIONS

(57) Abstract: Gram negative bacterial virulence genes are identified, thereby allowing the identification of novel anti-bacterial agents that target these virulence genes and their products, and the provision of novel gram negative bacterial mutants useful in vaccines.

ANTI-BACTERIAL VACCINE COMPOSITIONS

This application is a continuation-in-part of U.S. Patent Application Serial No: 09/545,199, filed April 6, 2000, which claims benefit of U.S. Provisional Patent Application Serial Nos. 60/153,453, filed September 10, 1999 and 60/128,689, filed April 9, 1999.

FIELD OF THE INVENTION

The present invention relates generally to the identification of genes responsible for virulence of *Pasteurellaceae* bacteria, thereby allowing for production of novel attenuated mutant strains useful in vaccines and identification of new antibacterial agents that target the virulence genes and their products.

BACKGROUND OF THE INVENTION

The family Pasteurellaceae encompasses several significant pathogens that infect a wide variety of animals. In addition to P. multocida, prominent members of the family include Pasteurella (Mannheimia) haemolytica, Actinobacillus pleuropneumoniae and Haemophilus somnus. P. multocida is a gram-negative, nonmotile coccobacillus which is found in the normal flora of many wild and domestic animals and is known to cause disease in numerous animal species worldwide [Biberstein, In M. Kilian, W. Frederickson, and E. L. Biberstein (ed.), Haemophilus, Pasteurella, and Actinobacillus. Academic Press, London, p. 61-73 (1981)]. The disease manifestations following infection include septicemias, bronchopneumonias, rhinitis, and wound infections [Reviewed in Shewen, et al., In C. L. Gyles and C. O. Thoen (ed.), Pathogenesis of Bacterial Infections in Animals. Iowa State University Press, Ames, p. 216-225 (1993), incorporated herein by reference].

Infection by P. multocida generally results from invasion during periods of stress, but transmission may also occur by aerosol or contact exposure, or via flea and tick vectors. In fowl, P. multocida infection gives rise to acute to peracute septicemia, particularly prevalent in domestic turkeys and wild waterfowl under stress conditions associated with overcrowding, laying, molting, or severe

elimatic change. In cattle, a similar hemorrhagic septicemia follows infection and manifests conditions including high fever and depression, generally followed by quick death. Transmission is most likely through aerosol contact, but infection can also arise during periods of significant climatic change. In rabbits, infection gives rise to recurring purulent rhinitis, generally followed by conjunctivitis, otitis media, sinusitis, subcutaneous abscesses, and chronic bronchopneumonia. In severe infections, rabbit mortality arises from acute fibrinous bronchopneumonia, septicemia, or endotoxemia. Disease states normally arise during periods of stress. In pigs, common P. multocida disease states include atrophic rhinitis and bacterial pneumonia. Similar pneumonia conditions are also detected in dogs, cats, goats, and sheep. P. multocida is commonly detected in oral flora of many animals and is therefore a common contaminant in bite and scratch wounds.

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P. multocida strains are normally designated by capsular serogroup and somatic serotype. Five cansular serogroups (A. B. D. E. and F) and 16 somatic serotypes are distinguished by expression of characteristic heat-stable antigens. Most strains are host specific and rarely infect more than one or two animals. The existence of different serotypes presents a problem for vaccination because traditional killed whole cell bacteria normally provide only serotype-specific protection. However, it has been demonstrated that natural infection with one serotype can lead to immunological protection against multiple serotypes [Shewen, et al., In C. L. Gyles and C. O. Thoen (Ed.), Pathogenesis of Bacterial Infections in Animals. Iowa State University Press, Ames, p. 216-225 (1993)] and cross protection can also be stimulated by using inactivated bacteria grown in vivo [Rimler, et al., Am J Vet Res. 42:2117-2121 (1981)]. One live spontaneous mutant P. multocida strain has been utilized as a vaccine and has been shown to stimulate a strong immune response [Davis, Poultry Digest. 20:430-434 (1987), Schlink, et al., Avian Dis. 31(1):13-21 (1987)]. This attenuated strain, however, has been shown to revert to a virulent state or cause mortality if the vaccine recipient is stressed [Davis, Poultry Digest, 20:430-434 (1987), Schlink, et al., Avian Dis. 31(1):13-21 (1987)].

Another member of the Pasteurella family, A. pleuropneumoniae exhibits strict host specificity for swine and is the causative agent of highly contagious porcine pleuropneumonia. Infection normally arises in intensive breeding conditions, and is believed to occur by a direct mode of transmission. The disease is often fatal and, as a result, leads to severe economic loss in the swine producing industry. A. pleuropneumoniae infection may be chronic or acute, and infection is characterized by a hemorrhagic, necrotic bronchopneumonia with accompanying fibrinous pleuritis. To date, bacterial virulence has been attributed to structural proteins, including serotype-specific capsular polysaccharides, lipopolysaccharides, and surface proteins, as well as extracellular cytolytic toxins. Despite purification and, in some instances cloning, of these virulence factors, the exact role of these virulence factors in A. pleuropneumoniae infection is poorly understood.

Twelve serotypes of A. pleuropneumoniae have been identified based on antigenic differences in capsular polysaccharides and production of extracellular toxins. Serotypes 1, 5, and 7 are most relevant to A. pleuropneumoniae infection in the United States, while serotypes 1, 2, 5, 7, and 9 are predominant in Europe. There are at least three significant extracellular toxins of A. pleuropneumoniae that are members of the haemolysin family and are referred to as RTX toxins. RTX toxins are produced by many Gram negative bacteria, including E. coli, Proteus wulgarisa, and Pasteurella haemolytica, and the proteins generally share structural and functional characteristics. Toxins from the various serotypes differ, however, in host specificity, target cells, and biological activities.

The major A. pleuropneumoniae RTX toxins include ApxI, ApxII, and ApxIII. ApxI and ApxII have haemolytic activity, with ApxI being more potent. ApxIII shows no haemolytic activity, but is cytotoxic for alveolar macrophages and neutrophils. Most A. pleuropneumoniae serotypes produce two of these three toxins. For example, serotypes 1, 5, 9, and 11 express ApxI and ApxII, and serotypes 2, 3, 4, 6, and 8 express ApxII and ApxIII. Serotype 10, however, produces only ApxI, and serotypes 7 and 12 express only ApxII. Those A. pleuropneumoniae serotypes that produce both ApxI and ApxII are the most virulent strains of the bacteria.

The Apx toxins were demonstrated to be virulence factors in murine models and swine infection using randomly mutated wild type bacteria [Tascon, et al., Mol. Microbiol. 14:207-216 (1994)]. Other A. pleuropneumoniae mutants have also been generated with targeted mutagenesis to inactivate the gene encoding the AopA outer membrane virulence protein [Mulks and Buysee, Gene 165:61-66 (1995)].

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At least eleven serotypes (1, 2, 5-9, 12-14 and 16) have been demonstrated within Mannheimia [Pasteurella] haemolytica [Angen, et al., Vet Microbiol 65(4):283-90 (1999)], a Pasteurellaceae species which is responsible for serious outbreaks of acute pneumonia in neonatal, weaned, growing and adult lambs, calves, and goats [Ackermann, et al., Microbes Infect 2(9):1079-88 (2000)]. Transportation, viral infections, overcrowding, and other stressful conditions predispose animals to M. haemolytica infection [Ackermann, et al., supra.] The leukotoxin (Lkt) of M. haemolytica is believed to play a significant role in pathogenesis, causing cell lysis and apoptosis that lead to the lung pathology characteristic of bovine shipping fever [Highlander, et al., Infect Immun 68(7):3916-22 (2000)] as well as lung injury in bovine pneumonic pasteurellosis [Jeyaseelan, et al., Microb Pathog 30(2):59-69 (2001)]. Lkt is a pore-forming exotoxin that has the unique property of inducing cytolysis only in ruminant leukocytes and platelets [Jeyaseelan, et al., (2001), supra.]. Cytolysis of many cell types is mediated by arachidonic acid (AA) and its generation by phospholipases is regulated by G-protein-coupled receptors [Jevaseelan, et al., (2001) supra] Recent studies indicate that M. haemolytica Lkt binds to bovine CD18, the common subunit of all beta2 integrins [Jeyaseelan, et al., Infect Immun 68(1):72-9 (2000)]. It has also been shown that LFA-1 is a Lkt receptor, Lkt binding to LFA-1 is not target cell specific, Lkt binding to bovine LFA-1 correlates with calcium elevation and cytolysis. and bovine LFA-1 expression correlates with the magnitude of Lkt-induced target cell cytolysis [Jeyaseelan, et al., Infect Immun 68(1):72-9 (2000)].

In attempts to produce vaccine compositions, traditional killed whole cell bacteria have provided only serotype-specific protection [MacInnes and Smart, supra], however, it has been demonstrated that natural infection with a highly virulent

serotype can stimulate strong protective immunity against multiple serotypes [Nielsen, Nord Vet Med. 31:407-13 (1979), Nielsen, Nord Vet Med. 36:221-234 (1984), Nielsen, Can J Vet Res. 29:580-582 (1988), Nielsen, ACTA Vet Scand. 15:80-89 (1994)]. One defined live-attenuated vaccine strain producing an inactive form of the ApxII toxin has shown promise for cross protection in swine [Prideaux, et al., Infection & Immunity 67:1962-1966 (1999)], while other undefined live-attenuated mutants have also shown promise [Inzana, et al., Infect Immun. 61:1682-6, (1993), Paltineanu, et al., In International Pig Veterinary Society, 1992, p. 214, Ulrera, et al., In International Pig Veterinary Society, 1992, p. 213].

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Because of the problems associated with vaccine formulations comprising bacterial strains with undefined, spontaneous mutations, there exists a need in the art for rational construction of live attenuated bacterial strains for use in vaccines that will safely stimulate protective immunity against homologous and heterologous Pasteurellaceae serotypes. There further exists a need to identify attenuated bacterial strains and genes required for bacterial virulence, thereby facilitating development of methods to identify anti-bacterial agents.

SUMMARY OF THE INVENTION

In general, the present invention provides materials and methods for production and use of vaccine compositions comprising attenuated gram negative bacteria. In one aspect, vaccine compositions of the invention comprise attenuated species in the Pasteurellaceae family of bacteria, which is known in the art and described, in part, in Dewhirst, et al., J. Bacteriol. 174:2002-2013 (1992), incorporated herein by reference in its entirety. Species in the family include, but are not limited to, A. actinomycetemcomitans, A. capsulatus, A. equuli, A. lignieresii, A. pleuropneumoniae (H. pleuropneumoniae), A. seminis, A. suis (H. suis), A. ureae (p. ureae), A. capsulatus, Bisgaard taxon 11, H. aegyptius, H. aphrophilus, H. aphrophilus, H. haemolylicus, H. influenzae, H. paracuniculus, H. paragallinarum, H. parathaemolyticus, H. paraginfluenzae, (H. paraphrophilus), H.

paraphrohaemolyticus, H. paraphrophilus, H. parasuis, H. parasuis type 5, H. segnis, H. somnus, Haemophilus minor group, Haemophilus taxon C, P. aerogenes, P. anatis, P. avium (H. avium), P. canis, P. dagmatis, P. gallinarum, P. (Mannheimia) haemolytica, P. tehalosi (P. haemolytica biotype T), P. langaa, P. multocida, P. pneumotropica, P. stomatis, P. volantium (H. parainfluenzae), P. volantium, Pasteurella species A, Pasteurella species B, and Haemophilus paraphrohaemolyticus. Preferably, vaccine compositions comprise attenuated Pasteurella (Mannheimia) haemolytica, Actinobacillus pleuropneumoniae, Haemophilus somnus, or Pasteurella multocida bacteria. In a most preferred embodiment, vaccine compositions of the invention comprise attenuated Pasteurella multocida and A. plueropneumoniae bacterial strains.

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One aspect of the invention provides gram negative bacterial organisms containing a functional mutation in a gene sequence represented by any one of SEO ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, or species homologs thereof, wherein the mutation inhibits or abolishes expression and/or biological activity of an encoded gene product (i.e., the polypeptide encoded by a gene); said functional mutation resulting in attenuated virulence of the bacterial strain. Functional mutations that modulate (i.e., increase or decrease) expression and/or biological activity of a gene product include insertions or deletions in the protein coding region of the gene itself or in sequences responsible for, or involved in, control of gene expression. Deletion mutants include those wherein all or part of a specific gene sequence is deleted. Also contemplated are compositions, and preferably vaccine compositions, comprising mutated and attenuated gram negative bacterial organisms, optionally comprising a suitable adjuvant and/or a pharmaceutically acceptable diluent or carrier. In order for a modified strain to be effective in a vaccine formulation, the attenuation must be significant enough to

prevent the pathogen from evoking severe clinical symptoms, but also insignificant enough to allow limited replication and growth of the bacteria in the host.

The invention also provides polynucleotides encoding gene products that are required for virulence in gram negative bacteria. Polynucleotides of the 5 invention include DNA, such as complementary DNA, genomic DNA including complementary or anti-sense DNA, and wholly or partially synthesized DNA; RNA. including sense and antisense strands; and peptide nucleic acids as described, for example in Corey, TIBTECH 15:224-229 (1997). Virulence gene polynucleotides of the invention include those set forth in SEO ID NOs:1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 10 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, or species homologs thereof, polynucleotides encoding a virulence gene product encoded by a polynucleotide of SEQ ID NOs: 1, 3, 15 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, or a species homolog thereof, and polynucleotide that hybridize, under moderately to highly stringent conditions, to the noncoding strand (or complement) of any one of the polynucleotides 20 set out in SEO ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 25 174, or species homologs thereof. The invention therefore comprehends gene sequences from Pasteurellaceae set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162,

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gram negative bacterial organisms, including naturally occurring (*i.e.*, species homologs) and artificially induced variants thereof. The invention also comprehends polynucleotides which encode polypeptides deduced from any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 164, 166, 168, 170, 172, and 174, and species homologs thereof. Knowledge of the sequence of a polynucleotide of the invention makes readily available every possible fragment of that polynucleotide. The invention therefore provides fragments of a polynucleotide of the invention.

The invention further embraces expression constructs comprising polynucleotides of the invention. Host cells transformed, transfected or electroporated with a polynucleotide of the invention are also contemplated. The invention provides methods to produce a polypeptide encoded by a polynucleotide of the invention comprising the steps of growing a host cell of the invention under conditions that permit, and preferably promote, expression of a gene product encoded by the polynucleotide, and isolating the gene product from the host cell or the medium of its growth.

Identification of polynucleotides of the invention makes available the encoded polypeptides. Polypeptides of the invention include full length and fragment, or truncated, proteins; variants thereof; fusion, or chimeric proteins; and analogs, including those wherein conservative amino acid substitutions have been introduced into wild-type polypeptides. Antibodies that specifically recognize polypeptides of the invention are also provided, and include monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted antibodies, as well as compounds that include CDR sequences which specifically recognize a polypeptide of the invention. The invention also provides anti-idiotype antibodies immunospecific for antibodies of the invention.

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According to another aspect of the invention, methods are provided for identifying novel anti-bacterial agents that modulate the function of gram negative bacteria virulence genes or gene products. Methods of the invention include screening potential agents for the ability to interfere with expression of virulence gene products encoded by the DNA sequences set forth in any one of SEO ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, or species homologs . thereof, or screening potential agents for the ability to interfere with biological function of a bacterial gene product encoded in whole or in part by a DNA sequence set forth in any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, species homologs thereof, or the complementary strand thereof. followed by identifying agents that provide positive results in such screening assays. In particular, agents that interfere with the expression of virulence gene products include anti-sense polynucleotides and ribozymes that are complementary to the virulence gene sequences. The invention further embraces methods to modulate transcription of gene products of the invention through use of oligonucleotide-directed triplet helix formation.

Agents that interfere with the function of virulence gene products include variants of virulence gene products, binding partners of the virulence gene products and variants of such binding partners, and enzyme inhibitors (where the product is an enzyme).

Novel anti-bacterial agents identified by the methods described herein are provided, as well as methods for treating a subject suffering from infection with gram negative bacteria involving administration of such novel anti-bacterial agents in an amount effective to reduce bacterial presence.

Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently prepared embodiments thereof.

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DETAILED DESCRIPTION OF THE INVENTION

"Virulence genes," as used herein, are genes whose function or products are required for successful establishment and/or maintenance of bacterial infection in a host animal. Thus, virulence genes and/or the proteins encoded thereby are involved in pathogenesis in the host organism, but may not be necessary for growth.

"Signature-tagged mutagenesis (STM)," as used herein, is a method generally described in International Patent Publication No. WO 96/17951, incorporated herein by reference, and includes, for example, a method for identifying bacterial genes required for virulence in a murine model of bacteremia. In this method, bacterial strains that each have a random mutation in the genome are produced using transposon integration; each insertional mutation carries a different DNA signature tag which allows mutants to be differentiated from each other. The tags comprise 40 bp variable central regions flanked by invariant "arms" of 20 bp which allow the central portions to be co-amplified by polymerase chain reaction (PCR). Tagged mutant strains are assembled in microtiter dishes, then combined to form the "inoculum pool" for infection studies. At an appropriate time after inoculation, bacteria are isolated from the animal and pooled to form the "recovered pool." The tags in the recovered pool and the tags in the inoculum pool are separately amplified, labeled, and then used to probe filters arrayed with all of the different tags representing the mutants in the inoculum. Mutant strains with attenuated virulence are those which cannot be recovered from the infected animal, i.e., strains with tags that give hybridization signals when probed with tags from the inoculum pool but not when probed with tags from the recovered pool. In a variation of this method, nonradioactive detection methods such as chemiluminescence can be used

Signature-tagged mutagenesis allows a large number of insertional mutant strains to be screened simultaneously in a single animal for loss of virulence. Screening nineteen pools of mutant P. multocida strains resulted in the identification of more than 60 strains with reduced virulence, many of which were confirmed to be attenuated in virulence by subsequent determination of an approximate LD₅₀ for the individual mutants. Screening of A. pleuropneumoniae mutants resulted in identification of more than 100 strains having mutations in 35 different genes. Of these, mutations in 22 genes results in significantly attenuated A. pleuropneumoniae strains. The nucleotide sequence of the open reading frame disrupted by the transposon insertion was determined by sequencing both strands and an encoded amino acid sequence was deduced. Novelty of both the polynucleotide and amino acid sequences was determined by comparison of the sequences with DNA and protein database sequences. Knowledge of the virulence genes in these species permitted identification of species homologs in P. (Mannheimia) haemolytica.

The identification of bacterial, and more particularly *P. multocida A. pleuropneumoniae* and *P. (Mannheimia) haemolytica* virulence genes provides for microorganisms exhibiting reduced virulence (*i.e.*, attenuated strains), which are useful in vaccines. Such microorganisms include *Pasteurellaceae* mutants containing at least one functional mutation inactivating a gene represented by any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174. The worker of ordinary skill in the art will easilize that a "functional mutation" may occur in protein coding regions of a gene of the invention, as well as in regulatory regions that modulate transcription of the virulence gene RNA.

The worker of ordinary skill will also appreciate that attenuated P. multocida, A. pleuropneumoniae and P. (Mannheimia) haemolytica strains of the invention include those bearing more than one functional mutation. More than one mutation may result in additive or synergistic degrees of attenuation. Multiple

mutations can be prepared by design or may fortuitously arise from a deletion event originally intended to introduce a single mutation. An example of an attenuated strain with multiple deletions is a Salmonella typhimurium strain wherein the cya and crp genes are functionally deleted. This mutant S. typhimurium strain has shown promise as a live vaccine.

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Identification of virulence genes in P. multocida, A. pleuropneumoniae and P. (Mannheimia) haemolytica can provide information regarding similar genes in other pathogenic species. As an example, identification of the aroA gene led to identification of conserved genes in a diverse number of pathogens, including Aeromonas hydrophila, Aeromonas salmonicida, Salmonella typhimurium, Salmonella enteritidis, Salmonella dublin, Salmonella gallanerum, Bordella pertussis, Yersinia entericolitica, Neisseria gonorrhoeae, and Bacillus anthracis. In many of these species, attenuated bacterial strains bearing mutations in the aroA gene have proven to be effective in vaccine formulations. Using the virulence genes sequences identified in P. multocida, similar or homologous genes can be identified in other organisms, particularly within the Pasteurella family, as well as A. pleuropneumoniae, P. (Mannheimia) haemolytica, and Haemophilus somnus. Likewise, identification of A. pleuropneumoniae virulence genes can permit identification of related genes in other organisms. Southern hybridization using the P. multocida, A. pleuropneumoniae and P. (Mannheimia) haemolytica genes as probes can identify these related genes in chromosomal libraries derived from other organisms. Alternatively, PCR can be equally effective in gene identification across species boundaries. As still another alternative, complementation of, for example, a P. inultocida mutant with a chromosomal library from other species can also be used to identify genes having the same or related virulence activity. Identification of related virulence genes can therefore lead to production of an attenuated strain of the other organism which can be useful as still another vaccine formulation. Examples of P. multocida genes that have been demonstrated to exist in other species (e.g. P. (Mannheimia) haemolytica. A. pleuropneumoniae and H. sonnus) include genes exbB, ainG, pnp. guaB and vigF.

Attenuated *P. multocida* strains identified using STM are insertional mutants wherein a virulence gene has been rendered non-functional through insertion of transposon sequences in either the open reading frame or regulatory DNA sequences. These insertional mutants still contain all of the genetic information required for bacterial virulence and can possibly revert to a pathogenic state by deletion of the inserted transposon. Therefore, in preparing a vaccine formulation, it is desirable to take the information gleaned from the attenuated strain and create a deletion mutant strain wherein some, most, or all of the virulence gene sequence is removed, thereby precluding the possibility that the bacteria will revert to a virulent state.

The vaccine properties of an attenuated insertional mutant identified using STM are expected to be the same or similar to those of a bacteria bearing a deletion in the same gene. However, it is possible that an insertion mutation may exert "polar" effects on adjoining gene sequences, and as a result, the insertion mutant may possess characteristic distinct from a mutant strain with a deletion in the same gene sequence. Deletion mutants can be constructed using any of a number of techniques well known and routinely practiced in the art.

In one example, a strategy using counterselectable markers can be employed which has commonly been utilized to delete genes in many bacteria. For a review, see, for example, Reyrat, et al., Infection and Immunity 66:4011-4017 (1998), incorporated herein by reference. In this technique, a double selection strategy is often employed wherein a plasmid is constructed encoding both a selectable and counterselectable marker, with flanking DNA sequences derived from both sides of the desired deletion. The selectable marker is used to select for bacteria in which the plasmid has integrated into the genome in the appropriate location and manner. The counterselecteable marker is used to select for the very small percentage of bacteria that have spontaneously eliminated the integrated plasmid. A fraction of these bacteria will then contain only the desired deletion with no other foreign DNA present. The key to the use of this technique is the availability of a suitable counterselectable marker.

In another technique, the cre-lox system is used for site specific recombination of DNA. The system consists of 34 base pair lox sequences that are recognized by the bacterial cre recombinase gene. If the lox sites are present in the DNA in an appropriate orientation, DNA flanked by the lox sites will be excised by the cre recombinase, resulting in the deletion of all sequences except for one remaining copy of the lox sequence. Using standard recombination techniques, it is possible to delete the targeted gene of interest in the P. multocida, A. pleuropneumoniae or P. (Mannheimia) haemolytica genome and to replace it with a selectable marker (e.g., a gene coding for kanamycin resistance) that is flanked by the lox sites. Transient expression (by electroporation of a suicide plasmid containing the cre gene under control of a promoter that functions in P. multocida, A. pleuropneumoniae, or P. (Mannheimia) haemolytica) of the cre recombinase should result in efficient elimination of the lox flanked marker. This process would result in a mutant containing the desired deletion mutation and one copy of the lox sequences.

In another approach, it is possible to directly replace a desired deleted sequence in the *P. multocida*, *A. pleuropneumoniae* or *P. (Mannheimia) haemolytica* genome with a marker gene, such as green fluorescent protein (GFP), β-galactosidase, or luciferase. In this technique, DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide (non-replicating) vector for *P. multocida*, *A. pleuropneumoniae*, or *P. (Mannheimia) haemolytica*. An expression cassette, containing a promoter active in *P. multocida*, *A. pleuropneumoniae*, or *P. (Mannheimia) haemolytica* and the appropriate marker gene, is cloned between the flanking sequences. The plasmid is introduced into wild-type *P. multocida*, *A. pleuropneumoniae* or *P. (Mannheimia) haemolytica*. Bacteria that incorporate and express the marker gene (probably at a very low frequency) are isolated and examined for the appropriate recombination event (*i.e.*, replacement of the wild type gene with the marker gene).

The reduced virulence of these organisms and their immunogenicity
may be confirmed by administration to a subject animal. While it is possible for an
avirulent microorganism of the invention to be administered alone, one or more of

such mutant microorganisms are preferably administered in a vaccine composition containing suitable adjuvant(s) and pharmaceutically acceptable diluent(s) or carrier(s). The carrier(s) must be "acceptable" in the sense of being compatible with the avirulent microorganism of the invention and not deleterious to the subject to be immunized. Typically, the carriers will be water or saline which will be sterile and pyrogen free. The subject to be immunized is a subject needing protection from a disease caused by a virulent form of P. multocida, A. pleuropneumoniae, P. (Mannheimia) haemolytica or other pathogenic microorganisms.

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It will be appreciated that the vaccine of the invention may be useful in the fields of human medicine and veterinary medicine. Thus, the subject to be immunized may be a human or other animal, for example, farm animals including cows, sheep, pigs, horses, goats and poultry (e.g., chickens, turkeys, ducks and geese) companion animals such as dogs and cats; exotic and/or zoo animals; and laboratory animals including mice, rats, rabbits, guinea pigs, and hamsters.

The invention also provides polypeptides and corresponding polynucleotides required for P. multocida, A. pleuropneumoniae or P. (Mannheimia) haemolytica virulence. The invention includes both naturally occurring and non-naturally occurring polynucleotides and polypeptide products thereof. Naturally occurring virulence products include distinct gene and polypeptide species as well as corresponding species homologs expressed in organisms other than P. multocida, A. pleuropneumoniae, or P. (Mannheimia) haemolytica strains. Non-naturally occurring virulence products include variants of the naturally occurring products such as analogs and virulence products which include covalent modifications. In a preferred embodiment, the invention provides virulence polynucleotides comprising the sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 and species homologs thereof, and polypeptides having amino acids sequences encoded by the polynucleotides.

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The present invention provides novel purified and isolated P. multocida, A. pleuropneumoniae and P. (Mannheimia) haemolytica polynucleotides (e.g., DNA sequences and RNA transcripts, both sense and complementary antisense strands) encoding the bacterial virulence gene products. DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. Genomic DNA of the invention comprises the protein coding region for a polypeptide of the invention and includes variants that may be found in other bacterial strains of the same species. "Synthesized," as used herein and is understood in the art, refers to purely chemical, as opposed to enzymatic. methods for producing polynucleotides. "Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means. Preferred DNA sequences encoding P. multocida virulence gene products are set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, and species homologs thereof. Preferred A. pleuropneumoniae DNA sequences encoding virulence gene products are set out in SEQ ID NOs: 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof. Preferred P. (Mannheimia) haemolytica virulence gene products are set out in SEO ID NOs: 166, 168, 170, 172 and 174, and species homologs thereof. The worker of skill in the art will readily appreciate that the preferred DNA of the invention comprises a double stranded molecule, for example, molecules having the sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 and species homologs thereof, along with the complementary molecule (the "noncoding strand" or "complement") having a sequence deducible from the sequence of SEO ID NO: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53,

55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, according to Watson-Crick base pairing rules for DNA. Also preferred are polynucleotides encoding the gene products encoded by any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 and species homologs thereof. The invention further embraces species, preferably bacterial, homologs of the *P. multocida*, *A. pleuropneumoniae* and *P. (Mannheimia) haemolytica* DNA.

The polynucleotide sequence information provided by the invention makes possible the identification and isolation of polynucleotides encoding related bacterial virulence molecules by well known techniques including Southern and/or Northern hybridization, and polymerase chain reaction (PCR). Examples of related polynucleotides include polynucleotides encoding polypeptides homologous to a virulence gene product encoded by any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, and species homologs thereof, and structurally related polypeptides sharing one or more biological and/or physical properties of a virulence gene product of the invention.

The invention also embraces DNA sequences encoding bacterial gene products which hybridize under moderately to highly stringent conditions to the non-coding strand, or complement, of any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146,

148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172 and 174, and species homologs thereof. DNA sequences encoding virulence polypeptides which would hybridize thereto but for the degeneracy of the genetic code are contemplated by the invention. Exemplary high stringency conditions include a final wash in buffer comprising 0.2X SSC/0.1% SDS, at 65°C to 75°C, while exemplary moderate stringency conditions include a final wash in buffer comprising 2X SSC/0.1% SDS, at 35°C to 45°C. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described in Ausubel, et al. (Eds.), Protocols in Molecular Biology, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, et al., (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

Autonomously replicating recombinant expression constructions such as plasmid and viral DNA vectors incorporating virulence gene sequences are also provided. Expression constructs wherein virulence polypeptide-encoding polynucleotides are operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided. The virulence genes may be cloned by PCR, using P. multocida genomic DNA as the template. For ease of inserting the gene into expression vectors, PCR primers are chosen so that the PCR-amplified gene has a restriction enzyme site at the 5' end preceding the initiation codon ATG, and a restriction enzyme site at the 3' end after the termination codon TAG, TGA or TAA. If desirable, the codons in the gene are changed, without changing the amino acids, according to E. coli codon preference described by Grosjean and Fiers, Gene, 18:199-209 (1982), and Konigsberg and Godson, Proc. Natl. Acad. Sci. (USA), 80:687-691 (1983). Optimization of codon usage may lead to an increase in the expression of the gene product when produced in E. coli. If the gene product is to be produced extracellularly, either in the periplasm of

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E. coli or other bacteria, or into the cell culture medium, the gene is cloned without its initiation codon and placed into an expression vector behind a signal sequence.

According to another aspect of the invention, host cells are provided, including procaryotic and eukaryotic cells, either stably or transiently transformed, transfected, or electroporated with polynucleotide sequences of the invention in a manner which permits expression of virulence polypeptides of the invention. Expression systems of the invention include bacterial, yeast, fungal, viral. invertebrate, and mammalian cells systems. Host cells of the invention are a valuable source of immunogen for development of antibodies specifically immunoreactive with the virulence gene product. Host cells of the invention are conspicuously useful in methods for large scale production of virulence polypeptides wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by, for example, immunoaffinity purification or any of the multitude of purification techniques well known and routinely practiced in the art. Any suitable host cell may be used for expression of the gene product, such as E. coli, other bacteria, including P. multocida. Bacillus and S. aureus, yeast, including Pichia pastoris and Saccharomyces cerevisiae, insect cells, or mammalian cells, including CHO cells, utilizing suitable vectors known in the art. Proteins may be produced directly or fused to a peptide or polypeptide, and either intracellularly or extracellularly by secretion into the periplasmic space of a bacterial cell or into the cell culture medium. Secretion of a protein requires a signal peptide (also known as pre-sequence); a number of signal sequences from prokaryotes and eukaryotes are known to function for the secretion of recombinant proteins. During the protein secretion process, the signal peptide is removed by signal peptidase to yield the mature protein.

To simplify the protein purification process, a purification tag may be added either at the 5' or 3' end of the gene coding sequence. Commonly used purification tags include a stretch of six histidine residues (U.S. Patent Nos. 5,284,933 and 5,310,663), a streptavidin-affinity tag described by Schmidt and Skerra, Protein Engineering, 6:109-122 (1993), a FLAG peptide [Hopp et al., Biotechnology, 6:1205-

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1210 (1988)], glutathione S-transferase [Smith and Johnson, Gene, 67:31-40 (1988)], and thioredoxin [LaVallie et al., Bio/Technology, 11:187-193 (1993)]. To remove these peptide or polypeptides, a proteolytic cleavage recognition site may be inserted at the fusion junction. Commonly used proteases are factor Xa, thrombin, and enterokinase.

The invention also provides purified and isolated P. multocida, A. pleuropneumoniae and P. (Mannheimia) haemolytica virulence polypeptides encoded by a polynucleotide of the invention. Presently preferred are polypeptides comprising the amino acid sequences encoded by any one of the polynucleotides set out in SEO ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 164, 166, 168, 170, 172 and 174, and species homologs thereof. The invention embraces virulence polypeptides encoded by a DNA selected from the group consisting of: a) the DNA sequence set out in any one of SEO ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 164, 166, 168, 170, 172, and 174 and species homologs thereof; b) DNA molecules encoding P. multocida, A. pleuropneumoniae or P. (Mannheimia) haemolytica. polypeptides encoded by any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 164, 166, 168, 170, 172, and 174, and species homologs thereof; and c) a DNA molecule, encoding a virulence gene product, that hybridizes under moderately stringent conditions to the DNA of (a) or (b).

The invention also embraces polypeptides that have at least about 99%, at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 65%, at least about 60%, at least about 65%, at least about 60%, at least about 65%, at least 65

about 55%, and at least about 50% identity and/or homology to the preferred polypeptides of the invention. Percent amino acid sequence "identity" with respect to the preferred polypeptides of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the virulence gene product sequence after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent sequence "homology" with respect to the preferred polypeptides of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in one of the virulence polypeptide sequences after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and also considering any conservative substitutions as part of the sequence identity. Conservative substitutions can be defined as set out in Tables A and B.

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Table A
Conservative Substitutions I

	SIDE CHAIN	CHARACTERISTIC	AMINO ACID
20	Aliphatic	Non-polar	GAP
			ILV
		Polar - uncharged	CSTM
			ΝQ
		Polar - charged	DE
25		-	ΚR
	Aromatic		HFWY
	Other		NODE

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Polypeptides of the invention may be isolated from natural bacterial cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving host cells of the invention. Virulence gene products of the invention may be full length polypeptides, biologically active fragments, or variants thereof which retain specific biological or immunological activity. Variants may comprise virulence polypeptide analogs wherein one or more

of the specified (i.e., naturally encoded) amino acids is deleted or replaced or wherein one or more non-specified amino acids are added: (1) without loss of one or more of the biological activities or immunological characteristics specific for the virulence gene product; or (2) with specific disablement of a particular biological activity of the virulence gene product. Deletion variants contemplated also include fragments lacking portions of the polypeptide not essential for biological activity, and insertion variants include fusion polypeptides in which the wild-type polypeptide or fragment thereof have been fused to another polypeptide.

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Variant virulence polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention. Conservative substitutions are recognized in the art to classify amino acids according to their related physical properties and can be defined as set out in Table A (from WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96). Alternatively, conservative amino acids can be grouped as defined in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77] as set out in Table B.

Table B
Conservative Substitutions II

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic)	
A. Aliphatic:	ALIVP
B. Aromatic:	F W
C. Sulfur-containing:	M
D. Borderline:	G
Uncharged-polar	
A. Hydroxyl:	STY
B. Amides:	NQ
C. Sulfhydryl:	C `
D. Borderline:	G
Positively Charged (Basic):	KRH
Negatively Charged (Acidic):	DE
	CHARACTERISTIC Non-polar (hydrophobic) A. Aliphatic: B. Aromatic: C. Sulfur-containing: D. Borderline: Uncharged-polar A. Hydroxyl: B. Amides: C. Sulfhydryl: D. Borderline: Positively Charged (Basic):

Variant virulence products of the invention include mature virulence gene products, i.e., wherein leader or signal sequences are removed, having additional amino terminal residues. Virulence gene products having an additional methionine residue at position -1 are contemplated, as are virulence products having additional methionine and lysine residues at positions -2 and -1. Variants of these types are particularly useful for recombinant protein production in bacterial cell types. Variants of the invention also include gene products wherein amino terminal sequences derived from other proteins have been introduced, as well as variants comprising amino terminal sequences that are not found in naturally occurring proteins.

The invention also embraces variant polypeptides having additional amino acid residues which result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as a fusion protein with glutathione-S-transferase (GST) provide the desired polypeptide having an additional glycine residue at position -1 following cleavage of the GST component from the desired polypeptide. Variants which result from expression using other vector systems are also contemplated.

Also comprehended by the present invention are antibodies (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, humanized, human, and CDR-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention) and other binding proteins specific for virulence gene products or fragments thereof. The term "specific for" indicates that the variable regions of the antibodies of the invention recognize and bind a virulence polypeptide exclusively (i.e., are able to distinguish a single virulence polypeptides from related virulence polypeptides despite sequence identity, homology, or similarity found in the family of polypeptides), but may also interact with other proteins (for example, S. aureus protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see

Harlow et al. (Eds), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY (1988), Chapter 6. Antibodies that recognize and bind fragments of the virulence polypeptides of the invention are also contemplated, provided that the antibodies are first and foremost specific for, as defined above, a virulence polypeptide of the invention from which the fragment was derived.

The DNA and amino acid sequence information provided by the present invention also makes possible the systematic analysis of the structure and function of the virulence genes and their encoded gene products. Knowledge of a polynucleotide encoding a virulence gene product of the invention also makes available anti-sense polynucleotides which recognize and hybridize to polynucleotides encoding a virulence polypeptide of the invention. Full length and fragment anti-sense polynucleotides are provided. The worker of ordinary skill will appreciate that fragment anti-sense molecules of the invention include (i) those which specifically recognize and hybridize to a specific RNA (as determined by sequence comparison of DNA encoding a virulence polypeptide of the invention to DNA encoding other known molecules) as well as (ii) those which recognize and hybridize to RNA encoding variants of the family of virulence proteins. Antisense polynucleotides that hybridize to RNA encoding other members of the virulence family of proteins are also identifiable through sequence comparison to identify characteristic, or signature, sequences for the family of molecules.

The invention further contemplates methods to modulate gene expression through use of ribozymes. For a review, see Gibson and Shillitoe, Mol. Biotech. 7:125-137 (1997). Ribozyme technology can be utilized to inhibit translation of mRNA in a sequence specific manner through (i) the hybridization of a complementary RNA to a target mRNA and (ii) cleavage of the hybridized mRNA through nuclease activity inherent to the complementary strand. Ribozymes can be identified by empirical methods but more preferably are specifically designed based on accessible sites on the target mRNA [Bramlage, et al., Trends in Biotech 16:434-438 (1998)]. Delivery of ribozymes to target cells can be accomplished using either

exogenous or endogenous delivery techniques well known and routinely practiced in the art. Exogenous delivery methods can include use of targeting liposomes or direct local injection. Endogenous methods include use of viral vectors and non-viral plasmids.

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Ribozymes can specifically modulate expression of virulence genes when designed to be complementary to regions unique to a polynucleotide encoding a virulence gene product. "Specifically modulate" therefore is intended to mean that ribozymes of the invention recognizes only a single polynucleotide. Similarly, ribozymes can be designed to modulate expression of all or some of a family of proteins. Ribozymes of this type are designed to recognize polynucleotide sequences conserved in all or some of the polynucleotides which encode the family of proteins.

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The invention further embraces methods to modulate transcription of a virulence gene of the invention through use of oligonucleotide-directed triplet helix formation. For a review, see Lavrovsky, et al., Biochem. Mol. Med. 62:11-22 (1997). Triplet helix formation is accomplished using sequence specific oligonucleotides which hybridize to double stranded DNA in the major groove as defined in the Watson-Crick model. Hybridization of a sequence specific oligonucleotide can thereafter modulate activity of DNA-binding proteins, including, for example, transcription factors and polymerases. Preferred target sequences for hybridization include transcriptional regulatory regions that modulate virulence gene product expression. Oligonucleotides which are capable of triplet helix formation are also useful for site-specific covalent modification of target DNA sequences.

Oligonucleotides useful for covalent modification are coupled to various DNA damazing agents as described in Lavrovsky, et al. [supra].

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The identification of *P. multocida*, *A. pleuropneumoniae* and *P.*(Mannheimia) haemolytica virulence genes renders the genes and gene products useful in methods for identifying anti-bacterial agents. Such methods include assaying potential agents for the ability to interfere with expression of virulence gene products represented by the DNA sequences set forth in any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68,

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70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 and species homologs thereof (i.e., the genes represented by DNA sequences of SEO ID NOS: 1, 3, 7, 9, 11, 5 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 encode the virulence gene product, or the DNA sequences of SEO ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 are adjacent the gene encoding the virulence gene product, or are involved in regulation of expression of the virulence gene product), or assaying potential agents for the ability to interfere with the function of a bacterial gene product encoded in whole or in part by a DNA sequence set forth in any one of SEO ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174, species homologs thereof, or the complementary strand thereof, followed by identifying agents that are positive in such assays. Polynucleotides and polyneptides useful in these assays include not only the genes and encoded polypeptides as disclosed herein, but also variants thereof that have substantially the same activity as the wild-type genes and polypeptides.

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The virulence gene products produced by the methods described above are used in high throughput assays to screen for inhibitory agents. The sources for potential agents to be screened are chemical compound libraries, fermentation media of Streptomycetes, other bacteria and fungi, and cell extracts of plants and other vegetations. For proteins with known enzymatic activity, assays are established based

on the activity, and a large number of potential agents are screened for ability to inhibit the activity. For proteins that interact with another protein or nucleic acid, binding assays are established to measure such interaction directly, and the potential agents are screened for ability to inhibit the binding interaction.

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The use of different assays known in the art is contemplated according to this aspect of the invention. When the function of the virulence gene product is known or predicted by sequence similarity to a known gene product, potential inhibitors can be screened in enzymatic or other types of biological and/or biochemical assays keyed to the function and/or properties of the gene product. When the virulence gene product is known or predicted by sequence similarity to a known gene product to interact with another protein or nucleic acid, inhibitors of the interaction can be screened directly in binding assays. The invention contemplates a multitude of assays to screen and identify inhibitors of binding by the virulence gene product. In one example, the virulence gene product is immobilized and interaction with a binding partner is assessed in the presence and absence of a putative inhibitor compound. In another example, interaction between the virulence gene product and its binding partner is assessed in a solution assay, both in the presence and absence of a putative inhibitor compound. In both assays, an inhibitor is identified as a compound that decreases binding between the virulence gene product and its binding partner. Other assays are also contemplated in those instances wherein the virulence gene product binding partner is a protein. For example, variations of the di-hybrid assay are contemplated wherein an inhibitor of protein/protein interactions is identified by detection of a positive signal in a transformed or transfected host cell as described in PCT publication number WO 95/20652, published August 3, 1995.

Candidate inhibitors contemplated by the invention include compounds selected from libraries of potential inhibitors. There are a number of different libraries used for the identification of small molecule modulators, including: (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of structural analogs of known compounds or compounds that are

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identified as "hits" or "leads" via natural product screening. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal pentides. and variants (non-naturally occurring) thereof. For a review, see Science 282:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides, or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers, Curr. Opin. Biotechnol. 8:701-707 (1997). Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

Still other candidate inhibitors contemplated by the invention can be designed and include soluble forms of binding partners, as well as binding partners as chimeric, or fusion, proteins. Binding partners as used herein broadly encompasses antibodies, antibody fragments, and modified compounds comprising antibody domains that are immunospecific for the expression product of the identified virulence gene.

Other assays may be used when a binding partner (i.e., ligand) for the virulence gene product is not known, including assays that identify binding partners of the target protein through measuring direct binding of test binding partner to the target protein, and assays that identify binding partners of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields and Song, Nature, 340:245-246 (1989), and Fields and Sternglanz, Trends in Genetics, 10:286-292 (1994), both of

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which are incorporated herein by reference. The two-hybrid system is a genetic assay for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA-binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA-binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is generally performed in yeast. The assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNAbinding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the noncovalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. When the virulence gene product (the first protein, for example) is already known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different test agents are added to the system; the presence of an inhibitory agent results in lack of a reporter signal.

When the function of the virulence gene product is unknown and no ligands are known to bind the gene product, the yeast two-hybrid assay can also be used to identify proteins that bind to the gene product. In an assay to identify proteins that bind to the first protein (the target protein), a large number of hybrid genes each encoding different second proteins are produced and screened in the assay. Typically, the second protein is encoded by a pool of plasmids in which total cDNA or genomic DNA is ligated to the activation domain. This system is applicable to a wide variety

of proteins, and it is not even necessary to know the identity or function of the second binding protein. The system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

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Other assays may be used to search for agents that bind to the target protein. One such screening method to identify direct binding of test ligands to a target protein is described in U.S. Patent No. 5,585,277, incorporated herein by reference. This method relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a target protein (i.e., when the test ligand is a ligand of the target protein), the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence of a test ligand which binds the target protein, than in the absence of a ligand. Binding of the ligand to the target protein can be determined by any method which distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

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Another method for identifying ligands for a target protein is described in Wieboldt et al., Anal. Chem., 69:1683-1691 (1997), incorporated herein by reference. This technique screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target protein. Agents that bind to the target protein are separated from other library components by centrifugal ultrafiltration. The specifically selected molecules that are retained on the filter are subsequently liberated from the target protein and analyzed by HPLC and pneumatically assisted electrospray (ion spray) ionization mass spectroscopy. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for small molecule libraries.

The inhibitors/binders identified by the initial screens are evaluated for their effect on virulence in in vivo mouse models of P. multocida infections. Models of bacteremia, endocarditis, septic arthritis, soft tissue abscess, or pneumonia may be utilized. Models involving use of other animals are also comprehended by the invention. For example, rabbits can be challenged with a wild type P. multocida strain before or after administration of varying amounts of a putative inhibitor/binder compound. Control animals, administered only saline instead of putative inhibitor/binder compound provide a standard by which deterioration of the test animal can be determined. Other animal models include those described in the Animal and Plant Health Inspection Sevice, USDA, January 1, 1994 Edition, §§113.69-113.70; Panciera and Corstvet, Am. J. Vet. Res. 45:2532-2537; Ames, et al., Can. J. Comp. Med. 49:395-400 (1984); and Mukkur, Infection and Immunity 18:583-585 (1977). Inhibitors/binders that interfere with bacterial virulence are can prevent the establishment of an infection or reverse the outcome of an infection once it is established.

Any adjuvant known in the art may be used in the vaccine composition, including oil-based adjuvants such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant, mycolate-based adjuvants (e.g., trehalose dimycolate), bacterial lipopolysaccharide (LPS), peptidoglycans (i.e., mureins, mucopeptides, or glycoproteins such as N-Opaca, muramyl dipeptide [MDP], or MDP analogs), proteoglycans (e.g., extracted from Klebsiella pneumoniae), streptococcal preparations (e.g., OK432), BiostimTM (e.g., OIK2), the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminum hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic® polyols, the Ribi adjuvant system (see, for example GB-A-2 189 141), or interleukins, particularly those that stimulate cell mediated immunity. An alternative adjuvant consisting of extracts of Amycolata, a bacterial genus in the order Actinomycetales, has been described in U.S. Patent No. 4,877,612. Additionally, proprietary adjuvant mixtures are commercially available. The adjuvant used will depend, in part, on the

recipient organism. The amount of adjuvant to administer will depend on the type and size of animal. Optimal dosages may be readily determined by routine methods.

The vaccine compositions optionally may include vaccine-compatible pharmaceutically acceptable (i.e., sterile and non-toxic) liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

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The vaccine compositions can be packaged in forms convenient for delivery. The compositions can be enclosed within a capsule, caplet, sachet, cachet, gelatin, paper, or other container. These delivery forms are preferred when compatible with entry of the immunogenic composition into the recipient organism and, particularly, when the immunogenic composition is being delivered in unit dose form. The dosage units can be packaged, e.g., in tablets, capsules, suppositories or cachets.

The vaccine compositions may be introduced into the subject to be immunized by any conventional method including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, or subcutaneous injection; by oral, sublingual, nasal, anal, or vaginal, delivery. The treatment may consist of a single dose or a plurality of doses over a period of time.

The invention also comprehends use of an attenuated bacterial strain of the invention for manufacture of a vaccine medicament to prevent or alleviate bacterial infection and/or symptoms associated therewith. The invention also provides use of inhibitors of the invention for manufacture of a medicament to prevent or alleviate bacterial infection and/or symptoms associated therewith.

The present invention is illustrated by the following examples.

Example 1 describes constructions of *P. multocida* mutants. Example 2 relates to screening for *P. multocida* mutants. Example 3 addresses methods to determine

virulence of the *P. multocida* mutants. Example 4 describes cloning of *P. multocida* virulence genes. Example 5 addresses identification of genes in other species related to *P. multocida* virulence genes. Example 6 describes construction of *A. pleuropneumoniae* mutants. Example 7 addresses screening for attenuated *A. pleuropneumoniae* mutants. Example 8 relates to identification of *A. pleuropneumoniae* virulence genes. Example 9 describes competition challenge of *A. pleuropneumoniae* mutants and wild type bacteria. Example 10 characterizes *A. pleuropneumoniae* genes identified. Example 11 addresses efficacy of *A. pleuropneumoniae* mutant to protect against wild type bacterial challenge. Example 12 describes identification of species homolog virulence genes in *P. (Mannheimia) haemolytica*.

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Example 1 Construction of a Library of Tagged-Transposon P. multocida Mutants

A library of tagged-transposon mutants was constructed in parental vector pLOF/Km [Herrero, et al., J Bacteriol. 172:6557-67 (1990)] which has previously been demonstrated to be functional and random in P. multocida [Lee, et al., Vet Microbiol. 50:143-8 (1996)]. Plasmid pLOF/Km was constructed as a modification of suicide vector pGP704 and included a transposase gene under control of the Tac promoter as well as the mini-Tn10 transposable element encoding kanamycin resistance. Plasmid pTEF-1 was constructed as described below by modifying pLOF/Km to accept sequence tags which contained a semi-random [NK]₃₅ sequence.

Plasmid pLOF/Km was first modified to eliminate the unique KpnI restriction site in the multiple cloning region and then to introduce a new KpnI site in the mini-Tn10 region. The plasmid was digested with KpnI and the resulting overhanging ends were filled in with Klenow polymerase according to manufacturer's suggested protocol. Restriction digests and ligations described herein were performed according to manufacturer's suggested protocols (Gibco BRL, Gaithersburg, MD and Boehringer Mannheim, Indianapolis, IN). The blunt end product was self-ligated to

produce a plasmid designated pLOF/Km-KpnI which was transformed into E.coli DH5α: Apir for amplification. E.coli DH5α: (Apir φ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(r_x, m_e, supE44, relA1, deoR, Δ(lacZYA-argF)U169, was propagated at 37°C in Luria-Bertani (LB) medium. Plasmids were prepared using QlAGEN SpinPreps from QlAGEN Inc. (Santa Clarita, CA) and digested with S/iI which cuts at a unique site within the mini-Tn10 transposable element. A S/iI-Kpn1-S/iI adaptor was prepared by annealing oligonucleotides TEF1 (SEQ ID NO: 86) and TEF3 (SEQ ID NO: 87) and the resulting double-stranded adapter was ligated into the S/iI site to create plasmid pTEF-1. Oligonucleotides TEF1 and TEF3 (as well as all other oligonucleotides described herein) were synthesized by Genosys Biotechnologies (The Woodlands, TX).

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TEF3

TEF14

TEF1	5'-AGGCCGGTACCGGCCGCCT	SEQ ID NO: 86	

SEQ ID NO: 87

SEQ ID NO: 88

Unique sequence tags for insertion into the KpnI site of pTEF-1 were prepared as follows. PCR was carried out to generate double stranded DNA tags using a GeneAmp XL PCR Kit (PE Applied Biosystems, Foster City, CA) under conditions including 250 µM each dNTP, 1.5 mM Mg(OAc)₂, 100 pmol each primer TEF14 (SEQ ID NO: 88) and TEF15 (SEQ ID NO: 89), 1 ng TEF26 (SEQ ID NO: 90) as template DNA and 2.5 units recombinant Tth DNA Polymerase XL.

5'-CATGGTACCCATTCTAAC

5'-CGGCCGGTACCGGCCTAGG

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	TEF15	5'-CTAGGTACCTACAACCTC	SEQ ID NO: 89			
	TEF26		SEQ ID NO: 90			
	5'-CTAGGTACCTACAACCTCAAGCTT-[NK]35-					
30	AAGCTTGGTTAGAATGGGTACCATG					

Reaction conditions included an initial incubation at 95°C for one minute, followed by thirty cycles of 30 seconds at 95°C, 45 seconds at 45°C, and 15 seconds at 72°C, followed by a final incubation at 72°C for two minutes. The PCR products were digested with *Kpnl* and purified using a QIAGEN Nucleotide Removal Kit (QIAGEN, Inc., Chatsworth, GA) according to the manufacturer's suggested protocol. The unique tag sequences were ligated into the mini-Tn10 element of linearized pTEF-1, previously digested with *Kpnl* and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) using standard procedures. The resulting plasmid library was transformed into *E.coli* DH5α:λpir. Colony blot analysis was performed according to the DIG User's Guide (Boehringer-Mannheim) with hybridization and detection performed as follows.

Hybridizations were essentially performed according to the Genius Non-Radioactive User's Guide (Boehringer Mannheim Biochemicals), the product sheet for the DIG-PCR labeling kit (Boehringer Mannheim Biochemicals), and the product sheet for CSPD (Boehringer Mannheim Biochemicals). For preparation of probes, a 100 µl primary PCR reaction was set up using Amplitaq PCR buffer (PE Applied Biosystems), 200 µM dNTPs, 140 pmol each of primers TEF5 (SEQ ID NO: 91) and TEF6 (SEQ ID NO: 92), 2 mM MgCl₂, 2.5 units Amplitaq (PE Applied Biosystems) and 1 ne of plasmid DNA.

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TEF5 5'-TACCTACAACCTCAAGCT SEQ ID NO: 91

TEF6 5'-TACCCATTCTAACCAAGC SEQ ID NO: 92

Cycle conditions included an initial incubation at 95°C for two minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 45 seconds, 72°C for 15 seconds and a final incubation at 72°C for three minutes. The amplification products were separated using electrophoresis on a 2% - 3:1 NuSieve GTG (FMC BioProducts, Rockland, ME, USA):Agarose gel and the 109 bp product was excised and purified. Gel extractions were carried out using a OIAGEN Gel Extraction kit (QIAGEN).

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Approximately 15 ng of the primary product was labeled in a $50 \mu l$ PCR reaction using the DIG PCR Kit, 50 pmol each of primers TEF24 and TEF25, and a 1:1 mix of DIG Probe Synthesis Mix with 2 mM dNTP stock solution.

TEF24 5'-TACCTACAACCTCAAGCTT SEO ID NO: 93

TEF25 5'-TACCCATTCTAACCAAGCTT SEQ ID NO: 94

PCR conditions included an initial incubation at 95°C for four minutes, followed by 25 cycles of 95°C for 30 seconds, 50°C for 45 seconds, 72°C for 15 seconds and a final incubation at 72°C for three minutes. The labeled PCR product was digested with HindIII in a total reaction volume of 90 μ1 and purified from the constant primer arms using a 2% - 3:1 NuSieve GTG (FMC BioProducts): Agarose gel. The region containing the labeled variable tag was excised and the entire gel slice was dissolved and denatured in 10 ml of DIG EasvHvb at 95°C for ten minutes.

Dot blots were prepared using a Hybond [®]-N* membrane (Amersham-Pharmacia Biotech). Target DNA for each tag was prepared in 96 well plates using approximately 30 ng of PCR product. An equal volume of 0.1 N NaOH was added to denature the sample and each sample was applied to the membrane with minimal vacuum using a Minifold I™ Dot-Blot Apparatus from Schleicher and Schuell (Keene, NH, USA). Each well was washed with 150 µl of Neutralization Solution (0.5 M Tris /3 M NaCl, pH 7.5) and 150 µl of 2X SSC. Membranes were UV-crosslinked in a Stratalinker (Stratagene, La Jolla, CA, USA) and prehybridized for one hour in 20 mls DIG EasyHyb Buffer at 42°C. The denatured probe was added and hybridization carried out overnight at 42°C. The membrane was washed two times in 2X SSC containing 0.1% SDS for five minutes each wash. Two high stringency washes were performed in 50 ml of pre-warmed 0.1X SSC buffer containing 0.1% SDS at 68°C for 15 minutes before proceeding with standard Genius Detection protocols (Genius Manual).

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It is desirable to use a non-radioactive detection system for safety, lower cost, ease of use, and reduction of hazardous materials. In initial experiments using similar procedures previously described [Mei, et al., Mol Microbiol. 26:399-407 (1997)], unacceptable background levels of hybridization were obtained in negative controls. In order to decrease background, tag length was increased by 30 bp to a total of 70, amplification primers were lengthened to include all sequence flanking the variable region, a lower concentration of dig-dUTP was used, and the conserved sequences flanking the sequence tag region were removed by gel purification. Most significantly, PCR was used to generate [NK]₃₃, sequence tags as the target DNA in dot blots rather than the entire plasmids containing the tagged transposons after detecting background hybridization from the transposon itself. Using these modifications background was eliminated making chemiluminescent/non-radioactive screening more effective.

Approximately four hundred different transformants resulting from the ligation of pTEF-1 with the PCR generated sequence tags were screened by colony blot and the 96 strongest hybridizing colonies were assembled into microtiter plates for further use. Even though the likelihood of duplicated tags was very low, half of the plate of master tags was probed against the other to confirm that no tags were duplicated. The plasmids containing these tags were purified and transformed into E.coli S17-1:λpir (pir, recA, thi, pro, hsd, (r-m+), RP4-2, (Tc::Mu), (Km::Tn7), [TmpR], [SmR]), and the transformed bacteria propagated at 37°C in Luria-Bertani (LB) medium. Each of the 96 E.coli S17-1:λpir transformants containing the tagged plasmid pTEF-1 was used in conjugative matings to generate transposon mutants of P. multocida. P. multocida strain TF5 is a spontaneous nalidixic acid resistant mutant derived from UC6731, a bovine clinical isolate. P. multocida strains were grown on brain heart infusion (BHI) media (Difco Laboratories, Detroit, MI, USA) at 37°C and in 5% CO₂ when grown on plates. Matings were set up by growing each E.coli S17-1:λpir /pTEF1:[NK]₂₆ clone and the TF5 strain to late log phase. Fifty ul of culture for each tagged-pTEF-1 clone was mixed with 200 µl of the TF5 culture and 50 µl of each mating mixture was spotted onto 0.22 TM filters previously placed on BHI plates

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containing 100 mM IPTG and 10 mM MgSO₄. Following overnight incubation at 37°C with 5% CO₂, mating mixtures were washed off of each filter into 3 ml of PBS and 25 µl of each was plated onto BHIN⁵⁰K¹⁰⁰ plates. Following selective overnight growth, colonies were assembled into microtiter plates by toothpick transfer into 200 µl BHIN⁵⁰K¹⁰ making sure that each well in a microtiter plate always contained a transposon mutant with the same sequence tag. Following overnight growth, 50 µl of 75% glycerol was added to each well and plates were stored frozen at -80°C.

Nineteen pools were assembled by transferring the transposon mutants to microtiter plates making sure that each well contained a transposon mutant with the appropriate tag for that well. In other words, a specific well in each microtiter plate always contained a transposon mutant with the same sequence tag even though the location of the transposon within those mutants may be different.

Example 2 Murine Screening for Attenuated P. multocida Mutants

Nineteen pools of Pasteurella multocida transposon mutants were

screened using a murine model of septicemia. Frozen plates of pooled *P. multocida* transposon mutants were removed from -80°C storage and subcultured by transferring 10 µl from each well to a new 96 well round bottom plate (Corning Costar, Cambridge, MA, USA) containing 200 µl of brain heart infusion (DIFCO) with 50 µg/ml halidixic acid (Sigma) and 50 µg/ml kanamycin (Sigma) (BHIN⁵⁰K*⁵⁰). Plates were incubated without shaking overnight at 37°C in 5% CO₂. Overnight plates were subcultured by transferring 10 µl from each well to a new flat bottomed 96-well plate (Corning Costar) containing 100 µl of BHI per well and incubating at 37°C with shaking at approximately 150 rpm. The OD₅₄₀ was monitored using a micro-titer plate reader. At an OD₅₄₀ of approximately 0.2 to 0.25, each plate was pooled to form the "input pool" by combining 100 µl from each of the wells of the micro-titer plate. The culture was diluted appropriately in BHI to doses of approximately 10⁴, 10⁵, 10⁶ CFU/ml and 0.2 ml of each dilution was used to infect female 14-16 g BALB/c mice by intraperitoneal administration. At two days post-infection, one or two surviving mice were euthanized and the spleens harvested. The entire spleen was homogenized

in 1.0 ml sterile 0.9 % saline. Dilutions of the homogenate from 10⁻² to 10⁻³ were prepared and plated onto BHIN⁵⁰K. plates. Following overnight growth, at least 20,000 colonies were pooled in 10 mls BHI broth to form the "recovered pool" and 0.5 ml of the recovered pool was centrifuged at 3,500 X g and the pellet used to prepare genomic DNA according to a previously described protocol [Wilson, In F. M. Ausubel, et al., (ed.), Current Protocols in Molecular Biology, vol. 1. John Wiley and Sons, New York, p. 2.4.1-2.4.5. (1997)].

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Initial experiments with virulent wild-type P. multocida indicated that organisms could be recovered from the spleen, lungs, kidneys, and liver indicating a truly septicemic model of infection. Dot blots for both the "input" and "recovered" pools were performed as described in Example 1 and evaluated both by visual inspection and by semi-quantitative analysis. Hybridization was carried out as described in Example 1 except that 5 µg of genomic DNA from input and recovered pools was used as template. Semi-quantitative analysis indicates whether a significant reduction in a single clone has occurred. If a mutant is unable to survive within the host, then the recovered signal should be very low compared to the input signal yielding a high input/recovered ratio. Most mutants will grow as well in vivo as in vitro and therefore a ratio of their signals should be approximately equal to 1. Clones selected by quantitative analysis as being highly reduced in the recovered pool were selected for further study. Additional clones with questionable input/recovered ratios were also selected after visually evaluating films made from the dot blots.

Example 3 Determination of Virulence for P. multocida Candidate Mutants

Each potential mutant which exhibited reduced recovery from splenic tissue was isolated from the original pool plate and used individually in a challenge experiment to verify and roughly estimate the attenuation caused by the transposon mutation. Individual candidate mutants from in vivo screens were grown on Sheep Blood Agar plates overnight in 5% CO₂ at 37°C. Approximately six colonies of each mutant were inoculated into BHI broth and allowed to grow for six hours. Dilutions were prepared and five mice each were infected as described above with 10°, 10°, 10°

and 10³ CFU each. Attenuation was determined by comparing mortality after six days relative to the wild type. Surviving mice were presumed to be protected and then challenged with a dose of wild type *P. multocida* at a concentration approximately 200-fold greater than the LD₂₀ for the wild type strain. Survival rate was then determined for each challenged group of mice.

Results indicated that 62 of 120 potential transposon mutants were attenuated, having an approximate LD_{50} of at least 10 fold higher than the wild type strain. The clones and their approximate LD_{50} values are listed in Table 1. A control experiment with the wild type strain was run in parallel with each set of challenges and in all cases mortality in wild type-challenged groups was 100%.

In addition to LD₃₀ values, Table 1 also provides data from vaccination and challenge experiments. Briefly, groups of mice (n = 5 to 10) were vaccinated by intraperitoneal injection with the individual P. multocida strains shown in Table 1 at a dose that was approximately 200 times greater than the LD₃₀ of the virulent, wild type strain. Animals were observed for 28 days after which mortality figures were calculated.

Table 1

P. multocida Virulence Genes

Nucleotide SEQ ID NO:	Representative Isolate	PossibleGene Function	Vaccination # survivers/total	Challenge # survivors/total	LD ₅₀
	wild type		0/10		<10
23	PM1B1	guaB	10/10, 10/10, 10/10	9/10, 9/10	4.3 x 10
11	PM1D1	dsbB	10/10, 5/10	10/10, 5/5	8.4 x 10
3	PM1BD7	atpG	5/5, 10/10	10/10	>3 x 10
74	PM1BE11	yhcJ (HI0145)	10/10	5/10	>2 x 10
70	PM1BF6	yabK (H11020)	3/5, 8/10	9/9	>2 x 10
19	PM2G8	fhaC	4/5, 9/10	9/9	>4 x 105
76	PM3C9	yiaO (HI0146)	3/5		>6 x 105
118	PM3G11	UnkO	4/5, 10/10	10/10	>3 x 105
31	PM7B4	iroA (UnkB)	0/5		
17	PM4C6	fhaB (fhaB2)	2/5, 10/10, 9/10	10/10, 9/9	>3 x 106
9	PM4G10-T9	dnaA	4/5		>5 x 105
1	PM4D5-T5	atpB	5/5		>4 x 105
53	PM4D5-T1	UnkC2	5/5		>4 x 105
15	PM4F2	fhaB (fhaB1)	3/5, 6/10, 10/10	6/6, 10/10	>3 x 105
41	PM5F7	nweB	4/5		1 x 103
7	PM5E2	devB	0/5, 3/10	2/3	ND
68	PM6H5-T1	xylA	5/5		>3 x 105
78	PM6H8	yigF (HI0719)	5/5, 9/10	9/9	>3 x 105
108	PM7D12	pnp	5/5, 9/10	9/9	
51	PM8C1R1-T2	UnkC1	5/5		~6 x 105

Nucleotide	Representative	PossibleGene	Vaccination	Challenge	LD ₅₆
SEQ ID NO:	Isolate	Function	# survivors/total	# survivors/total	
37	PM8C1-T3	mgIB	5/5	-	-6 x 1
58	PM8C1R1-T6	UnkD1	5/5		~6 x 1
45	PM10H7	purF (H11207)	3/5, 8/10, 8/10	8/8, 8/8	>3 x 1i
25	PM10H10-T2	H11501	5/5		>1 x 1
72	PM11G8-T2	ygiK	5/5		>2.4 x *
21	PM11G8-T4	greA	5/5		>2.4 x
84	PM12H6	yyam (HI0687)	3/5, 0/10		-2.2 x 1
33	PM15G8-T2	kdtB	5/5		>1.2 x 1
116	PM15G8-T1	UnkK	5/5		>1.2 x 1
104	PM16G11-T1	hmbR	3/5		>1.9 x 1
29	PM16G11-T2	hxuC	3/5		>1.9 x 1
35	PM16H8	1gtC	5/5, 10/10	10/10	>2.4 x 1
80	PM16H3	ylcA (H10019)	5/5, 10/10		> 2.0 x 1
49	PM17H6-T1	sopE	4/5		-6 x 10
120	PM17H6	UnkP	4/5		~6 x 10
5	PM18F5-T8	cap5E	5/5		>2.4 x 1
82	PM18F5-T10	yojB (HI0345)	5/5		>2.4 x 1
13	PM19A1	exbB	5/5, 10/10	10/10	>1.2 x 1
112	PM19D4	rci	5/5, 8/10	8/8	~1.6 x 1
39	PM20A12	mioC (HI0669)	3/5, 8/10	8/8	-2 x 10
60	PM20C2 ·	UnkD2	5/5, 10/10	10/10	>8.2 x 1

Example 4
Cloning and Identification of Genes Required for *P. multocida* Virulence

Each transposon mutant which was verified to be attenuated was analyzed further to determine the identity of the disrupted open reading frame. DNA from each mutant was amplified, purified, and digested with restriction enzymes that were known not to cut within the transposon and generally produced 4-8 kb fragments that hybridized with the transposon. Using selection for kanamycin resistance encoded by the transposon, at least one fragment for each transposon mutant was cloned.

Southern hybridization with multiple restriction enzymes was performed for each attenuated mutant using a labeled 1.8 kb MluI fragment from pLOF/Km as a probe to identify a suitably sized fragment for cloning. The mini-Tn10 element and flanking DNA from each mutant was cloned into pUC19 and the flanking sequence determined using internal primers TEF32 and TEF40, primer walking and in some cases universal pUC-19 primers.

TEF-32 GGCAGAGCATTACGCTGAC SEQ ID NO: 95
TEF-40 GTACCGGCCAGGCGGCCACGCGTATTC SEO ID NO: 96

Sequencing reactions were performed using the BigDye™ Dye Terminator Chemistry kit from PE Applied Biosystems (Foster City, CA) and run on an ABI Prism 377 DNA Sequencer. Double stranded sequence for putative interrupted open reading frames was obtained for each clone. Sequencer 3.0 software (Genecodes, Corp., Ann Arbor, MI) was used to assemble and analyze sequence data. GCG programs [Devereux, et al., 1997. Wisconsin Package Version 9.0, 9.0 ed. Genetics Computer Group, Inc., Madison] were used to search for homologous sequences in currently available databases.

In 37% of the clones that were identified as being attenuated, there were multiple insertions of the mini-Tn10 transposable element. Each insertion including its flanking sequence was cloned individually into pGP704 and mated into the wild-type strain to produce new mutants of *P. multocida*, each carrying only one of the multiple original insertions. Individual mutants were retested individually to determine the insertion responsible for the attenuated phenotype. The nucleotide sequence of the disrupted, predicted open reading frame was determined by sequencing both strands, and the predicted amino acid sequence was used to search currently available databases for similar sequences. Sequences either matched known genes, unknown genes, and hypothetical open reading frames previously sequenced or did not match any previously identified sequence. For those genes having homology to previously identified sequences, potential functions were assigned as set out in Table 1

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Example 5 Identification of Related Genes in Other Species

In separate experiments, STM was also performed using Actinobacillus pleuropneumoniae (App). One of the App strains contained an insertion in a gene that was sequenced (SEQ ID NO: 97) and identified as a species homolog of the P. multocida atpG gene. This result suggested the presence in other bacterial species of

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homologs to previously unknown *P. multocida* genes that can also be mutated to produce attenuated strains of the other bacterial species for use in vaccine compositions. In order to determine if homologs of other *P. multocida* genes exists in other bacterial species, Southern hybridization was performed on genomic DNA from other species using the *A. pleuropneumoniae* atpG gene as a probe.

Actinobacillus pleuropneumoniae, Pasteurella haemolytica (Ph), P. multocida, and Haemophilus somnus (Hs) genomic DNA was isolated using the CTAB method and digested with EcoRl and HindIII for two hours at 37°C. Digested DNA was separated on a 0.7% agarose gel at 40V in TAE buffer overnight. The gel was immersed sequentially in 0.1 M HCL for 30 minutes, twice in 0.5 M NaOH/1.5 M NaCl for 15 minutes each, and twice in 2.5 M NaCl/1 M Tris, pH 7.5. The DNA was transferred to nitrocellulose membranes (Amersham Hybond N') overnight using 20X SSC buffer (3 M NaCl/0.3 M sodium citrate). The DNA was crosslinked to the membrane using a UV Stratalinker on autocrosslink setting (120 millijoules). The membrane was prehybridized in 5X SSC/1% blocking solution/0.1% sodium lauroyl sarcosine/0.02% SDS at 50°C for approximately seven hours and hybridized overnight at 50°C in the same solution containing a PCR generated atgG probe.

The probe was prepared using primers DEL-1389 (SEQ ID NO: 98) and TEF-46 (SEQ ID NO: 99) in a with a GeneAmp XL PCR kit in a GeneAmp PCR System 2400. Template was genomic *A. pleuropneumoniae* DNA.

DEL-1389	TCTCCATTCCCTTGCTGCGGCAGGG	SEQ ID NO: 98
TEF-46	GGAATTACAGCCGGATCCGGG	SEO ID NO: 99

The PCR was performed with an initial heating step at 94°C for five minutes, 30 cycles of denaturation t 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for three minutes, and a final extension step at 72°C for five minutes. The amplification products were separated on an agarose gel, purified using a QlAquick gel purification kit (QlAGEN), and labeled using a DIG-High Primer kit (Boehringer Mannheim). The blot was removed from the hybridization solution and rinsed in 2X

SSC and washed two times for five minutes each wash in the same buffer. The blot was then washed two times for 15 minutes each in 0.5X SSC at 60°C. Homologous bands were visualized using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

Single bands were detected in Pasteurella haemolytica, Haemophilus somnus and A. pleuropneumoniae using EcoRl digested DNA. Two bands were detected using EcoRi digested DNA from Pasteurella multocida.

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Example 6 Construction of a Library of Tagged-Transposon P. multocida Mutants

Transposon mutagenesis using pLOF/Km has previously been reported to be functional and random in A. pleuropneumoniae [Tascon, et al., J Bacteriol. 175:5717-22 (1993)]. To construct tagged transposon mutants of A. pleuropneumoniae, each of 96 E. coli S17-1:λpir transformants containing preselected tagged plasmids (pTEF-1:[NK]₃₅) was used in conjugative matings to generate transposon mutants of A. pleuropneumoniae strain AP225, a serotype I spontaneous nalidixic acid resistant mutant derived from an in vivo passaged ATCC 27088 strain. A. pleuropneumoniae strains were grown on Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, MI) media with 10 μg/ml B-nicotinamide adenine dinucleotide (V¹⁶), (Sigma, St. Louis, Missouri) at 37°C and in 5% CO₂ when grown on plates. E.coli S17-1:λpir (λpir, recA, thi, pro, hsdR(r_k-m_k+), RP4-2, (Tc^R::Mu), (Km^R::Tn7), [Tmp^R], [Sm^R]) was propagated at 37°C in Luria-Bertani (LB) medium. Antibiotics when necessary were used at 100 μg/ml ampicillin (Sigma), 50 μg/ml nalidixic acid (N⁵⁰)(Sigma), and 50 (K⁵⁰) or 100 (K¹⁰⁰) μg/ml of kanamycin (Sigma).

Matings were set up by growing each *E. coli* S17-1:λpir/pTEF1:[NK]₃₅ clone and the AP225 strain to late log phase. A 50 μl aliquot of culture for each tagged-pTEF-1 clone was mixed with 150 μl of the APP225 culture, and then 50 μl of each mating mixture was spotted onto 0.22 μM filters previously placed onto BHIV¹⁰ plates containing 100 μM IPTG and 10 mM MgSO₄. Following overnight incubation at 37°C with 5% CO₂, mating mixtures were washed off of each filter into 2 ml of PBS and 200 μl of each was plated onto BHIV¹⁰N⁵⁰K¹⁰⁰ plates. After selective

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overnight growth, colonies were assembled into microtiter plates by toothpick transfer into 200 μ l BHIV $^{10}N^{50}K^{50}$ making sure that each well in a microtiter plate always contained a transposon mutant with the same sequence tag. Following overnight growth, 50 μ l of 75% glycerol was added to each well and plates were stored frozen at $-80^{\circ}C$

APP does not appear to have as much bias towards multiple insertions of the mini-Tn10 element as did P. multocida. Only approximately 3% of the mutants were determined to contain multiple insertions, which is in agreement with the 4% previously reported [Tascon, et al., J Bacteriol. 175:5717-22 (1993)]. A problem in APP consisted of identifying numerous mutants (discussed below) containing insertions into 23S RNA regions: 28 total mutants with insertions into 13 unique sites. This may indicate that 23S RNA contains preferential insertion sites and that the growth of APP is affected by these insertions enough to result in differential survival within the host. Southern blot analysis using an APP 23S RNA probe suggests that APP may contain only three ribosomal operons as compared to five in H. influenzae [Fleischmann, et al., Science 269:496-512 (1995)] and seven complete operons in E. coli [Blattner, et al., Science 277:1453-1474 (1997)]. This site preference and its effect on growth rate may be a significant barrier to "saturation mutagenesis" since a significant number of clones will contain insertions into these rRNAs and large volume screening will be necessary to obtain additional unique attenuating mutations.

Example 7 Porcine Screening for Attenuated A. pleuropneumoniae Mutants

Twenty pools of A. pleuropneumoniae transposon mutants, containing a total of approximately 800 mutants, were screened using a porcine intratracheal infection model. Each pool was screened in two separate animals.

Frozen plates of pooled A. pleuropneumoniae transposon mutants were removed from -80°C storage and subcultured by transferring 20 µl from each well to a new 96 well round bottom plate (Corning Costar, Cambridge, MA, USA) containing 180 µl of BHIV¹⁰N²⁶K.²⁰. Plates were incubated without shaking overnight at 37°C in

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5% CO₂. Overnight plates were then subcultured by transferring 10 µl from each well to a new flat bottomed 96 well plate (Corning Costar) containing 100 µl of BHIV10 per well and incubating at 37°C with shaking at 150 rpm. The OD562 was monitored using a microtiter plate reader. At an OD of approximately 0.2 to 0.25, each plate was pooled to form the "input pool" by combining 100 ul from each of the wells of the microtiter plate. The culture was diluted appropriately in BHI to approximately 2 X 106 CFU/ml. For each diluted pool, 4.0 ml was used to infect 10-20 kg SPF pigs (Whiteshire-Hamroc, Albion, IN) by intratracheal administration using a tracheal tube. At approximately 20 hours post-infection, all surviving animals were euthanized and the lungs removed. Lavage was performed to recover surviving bacteria by infusing 150 mls of sterile PBS into the lungs, which were then massaged to distribute the fluid. The lavage fluid was recovered, and the process was repeated a second time. The lavage fluid was centrifuged at 450 x g for 10 minutes to separate out large debris. Supernatants were then centrifuged at 2,800 x g to pellet the bacteria. Pellets were resuspended in 5 mls BHI and plated in dilutions ranging from 10.2 to 10.5 onto BHIV10N50K50 plates. Following overnight growth, at least 100,000 colonies were pooled in 10 mls BHI broth to form the "recovered pools". A 0.7 ml portion of each recovered pool was used to prepare genomic DNA by the CTAB method [Wilson, In Ausubel, et al., (eds.), Current Protocols in Molecular Biology, vol. 1. John Wiley and Sons, New York, p. 2.4.1-2.4.5 (1997)].

Recovery from the animals routinely was in the $10^8\, \text{CFU}$ range from lung lavage.

Dot blots were performed and evaluated both by visual inspection and by semi-quantitative analysis as described previously. All hybridizations and detections were performed as described. Briefly, probes were prepared by a primary PCR amplification, followed by agarose gel purification of the desired product and secondary PCR amplification incorporating dig-dUTP. Oligonucleotides including TEF5, TEF6, TEF24, TEF25, TEF48 and TEF62, were synthesized by Genosys Biotechnologies (The Woodlands, TX). Primers TEF69, TEF65, and TEF66 were also used for inverse PCR reactions and sequencing.

TEF69	GACGTTTCCCGTTGAATATGGCTC	SEQ ID NO: 166
TEF65	GCCGGATCCGGGATCATATGACAAGA	SEQ ID NO: 167
TEF66	GACAAGATGTGTATCCACCTTAAC	SEQ ID NO: 168

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The labeled PCR product was then digested with HindIII to separate the constant primer arms from the unique tag region. The region containing the labeled variable tag was excised and the entire gel slice was then dissolved and denatured in DIG EasyHyb. Dot blots were prepared and detected using the standard CSPD detection protocol. Film exposures were made for visual evaluation, and luminescent counts per second (LCPS) were determined for each dot blot sample. The LCPS input / LCPS recovered ratio for each mutant was used to determine mutants likely to be attenuated.

Clones selected as being present in the input pool but highly reduced in the recovered pool were selected for further study. Additional clones with questionable input/recovered ratios were also selected after visually evaluating films made from the dot blots. A total of 110 clones were selected.

Example 8 Identification of A. pleuropneumoniae Virulence Genes

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A partial flanking sequence was determined for each of the 110 mutants by inverse PCR and direct product sequencing. Inverse PCR was used to generate flanking DNA products for direct sequencing as described above.

Sequencing reactions were performed using the BigDyetm Dye Terminator Chemistry kit from PE Applied Biosystems (Foster City, CA) and run on an ABI Prism 377 DNA Sequencer. Sequencher 3.0 software (Genecodes, Corp., Ann Arbor, MI) was used to assemble and analyze sequence data. GCG programs [Devereux and Haeberli, Wisconsin Package Version 9.0, 9.0 ed. Genetics Computer Group, Inc., Madison (1997)] were used to search for homologous sequences in currently available databases

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Table 2 shows the A. pleuropneumoniae genes identified and extent to which open reading frames were determinable. Sequence identification numbers are provided for nucleotide sequences as well as deduced amino acid sequences where located.

Table 2
A. pleuropneumoniae Open Reading Frames

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	Complete Open Re	ading Frame	NO Start Codon - Sto	p Codon
	atpH	SEQ ID NO: 134	dksA	SEQ ID NO: 136
10	aptG	SEQ ID NO: 132	dnaK	SEQ ID NO: 138
	exbB	SEQ ID NO: 140	HI0379	SEQ ID NO: 144
	OmpP5	SEQ ID NO: 152		
	OmpP5-2	SEQ ID NO: 150	NO Start Codon - NO	Stop Codon
	tig	SEQ ID NO: 160	pnp	SEQ ID NO: 154
15	fkpA	SEQ ID NO: 142	apvA-or 1	SEQ ID NO: 122
	hupA	SEQ ID NO: 146	apvA-or 2	SEQ ID NO: 124
	rpmF	SEQ ID NO: 158	apvB	SEQ ID NO: 126
	-		apvD	SEQ ID NO: 130
	Start Codon - NO S	top Codon		
20	lpdA	SEQ ID NO: 148	RNA or Noncoding S	equences
	potD	SEQ ID NO: 156	tRNA-leu	SEQ ID NO: 162
	yaeE	SEQ ID NO: 164	tRNA-glu	SEQ ID NO: 163
	apvC	SEO ID NO: 128	-	

The putative identities listed in Table 3 (below, Example 9) were assigned by comparison with bacterial databases. The 110 mutants represented 35 groups of unique transposon insertions. The number of different mutations per loci varied, with some clones always containing an insertion at a single site within an ORF to clones containing insertions within different sites of the same ORF. Three multiple insertions were detected in the 110 mutants screened as determined by production of multiple PCR bands and generation of multiple sequence electropherograms.

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Example 9 Competition Challenge of A. pleuropneumoniae Mutants with Wild Type APP225

A representative clone from each of the unique attenuated mutant groups identified above that was absent or highly reduced in the recovered population was isolated from the original pool plate and used in a competition challenge experiment with the wild type strain (AP225) to verify the relative attenuation caused by the transposon mutation. Mutant and wild type strains were grown in BHIV¹⁰ to an OD₅₀₀ of 0.6 – 0.9. Approximately 5.0 × 10⁶ CFU each of the wild type and mutant strains were added to 4 mls BHI. The total 4 ml dose was used infect a 10-20 kg SPF pig by intratracheal administration with a tracheal tube. At approximately 20 hours post-infection, all surviving animals were euthanized and the lungs removed. Lung lavages were performed as described above. Plate counts were carried out on BHIV¹⁶N⁵⁰ and BHIV¹⁶N⁵⁰K¹⁰⁰ to determine the relative numbers of wild type to mutant in both the input cultures and in the lung lavage samples. A Competitive Index (CI) was calculated as the [mutant CFU / wild type CFU]_{input} / [mutan

Of the 35 potential transposon mutants, 22 were significantly attenuated, having a competitive index (CI) of less than 0.2. A transposon mutant that did not seem to be attenuated based on the STM screening results was chosen from one of the pools as a positive control. This mutant had a CI in vivo of approximately 0.6. An in vitro competition was also done for this mutant resulting in a CI of 0.8. The mutant was subsequently determined to contain an insertion between 2 phenylalanine tRNA's.

Competitive indices for unique attenuated single-insertion mutants are listed in Table 3. Competitive indices for atpG, pnp, and exbB App mutants indicated that the mutants were unable to compete effectively with the wild type strains and were therefore attenuated.

Table 3
Virulence and Proposed Function of A. pleuropneumoniae Mutants

	Mutant	Similarity	Putative or Known Functions	C.I.
5	AP20A6			
,		atpH	ATP synthase	.009
	AP7F10	atpG_	ATP synthase	.013
	AP17C6	lpdA	dihydrolipoamide dehydrogenase	.039
	AP11E7	exbB	transport of iron compounds	.003,.003,.006
0	AP3H7	potD		
U			Spermidine/putrescine transport	.308
	AP8H6	OmpP5	Adhesin / OmpA homolog	.184
	AP18H8	OmpP5-2	Adhesin / OmpA homolog	.552
	AP13E9	tig	Peptidyl-prolyl isomerase	.050
	AP13C2	fkp∧	Peptidyl-prolyl isomerase	<.001
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	AP15C11	pnp	Polynucleotide phosphorylase	.032
	AP18F12	hupA	Histone - like protein	.001
	AP20F8	dksA	Dosage dependent suppressor of dnaK mutations	.075
	AP5G4	dnaK	Heat shock protein - molecular chaperone	.376
0				
	AP17C9	tRNA-leu	Protein Synthesis	.059
	AP5D6	tRNA-glu	Protein Synthesis	.055
	AP18B2	rpmF	Protein Synthesis	.112
5	AP10E7	yaeA	Unknown	.001
	AP19A5	HI0379	Unknown	.061
	AP10C10	аруА	Unknown	.157
	AP18F5	аруВ	Unknown	.103
	AP2A6	apvC	Unknown	.091
0	AP2C11	apvD	Unknown	.014

Accuracy of the CI appeared to be very good as the exbB mutant was competed within three different animals yielding CI's of 0.003, 0.003 and 0.006. The use of a Competitive Index number to assign attenuation based upon one competition in a large animal study was further confirmed based on preliminary vaccination results in pigs with 7 mutants (n=8) described below in Example 11.

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Example 10

Characterization of Attenuated A. pleuropneumoniae Virulence Genes

The A. pleuropneumoniae genes identified represent four broad functional classes: biosynthetic enzymes, cellular transport components, cellular regulation components and unknowns.

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The atpG gene, encoding the F1-γ subunit of the F₀F₁ H+-ATPase complex, can function in production of ATP or in the transport of protons by hydrolyzing ATP. A related atpG attenuated mutant was also identified in P. multocida. Another atp gene, atpH, that encodes the F₁ δ subunit was also identified. Phenotypes of atp mutants include non-adaptable acid-sensitivity phenotype [Foster, J Bacteriol. 173:6896-6902 (1991)], loss of virulence in Salmonella typhimurium [Garcia del Portillo, et al., Infect Immun. 61:4489-4492 (1993)] and P. multocida (above) and a reduction in both transformation frequencies and induction of competence regulatory genes in Haemophilus influenzae Rd [Gwinn, et al., J Bacteriol. 179:7315-20 (1997)].

LpdA is a dihydrolipoamide dehydrogenase that is a component of two enzymatic complexes: pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. While the relationship to virulence is unknown, production of LpdA is induced in Salmonella typhimurium when exposed to a bactericidal protein from human which may suggest that this induction may be involved in attempts to repair the outer membrane [Qi, et al., Mol Microbiol. 17:523-31 (1995)].

Transport of scarce compounds necessary for growth and survival are critical in vivo. ExbB is a part of the TonB transport complex [Hantke, and Zimmerman, Microbiology Letters. 49:31-35 (1981)], interacting with TonB in at least two distinct ways [Karlsson, et al., Mol Microbiol. 8:389-96 (1993), Karlsson, et al., Mol Microbiol. 8:379-88 (1993)]. Iron acquisition is essential for pathogens. In this work, attenuated exbB mutants in both APP and P. multocida have been identified. Several TonB-dependent iron receptors have been identified in other bacteria [Biswas, et al., Mol. Microbiol. 24:169-179 (1997), Braun, FEMS Microbiol Rev. 16:295-307 (1995), Elkins, et al., Infect Immun. 66:151-160 (1998), Occhino, et

al., Mol Microbiol. 29:1493-507 (1998), Stojiljkovic and Srinivasan, J Bacteriol.

179:805-12 (1997)]. A. pleuropneumoniae produces 2 transferrin-binding proteins, which likely depend on the ExbB/ExbD/TonB system, for acquisition of iron. PotD is a periplasmic binding protein that is required for spermidine (a polyamine) transport [Kashiwagi, et al., J Biol Chem. 268:19358-63 (1993)]. Another member of the Pasteurellaceae family, Pasteurella haemolytica, contains a homologue of potD (Lpp38) that is a major immunogen in convalescent or outer membrane protein vaccinated calves [Pandher and Murphy, Vet Microbiol. 51:331-41 (1996)]. In P. haemolytica, PotD appeared to be associated with both the inner and outer membranes. The role of PotD in virulence or in relationship to protective antibodies is unknown although previous work has shown potD mutants of Streptococcus pneumoniae to be attenuated [Polissi, et al., Infect. Immun. 66:5620-9 (1998)].

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Relatively few "classical virulence factors," such as adhesins or toxins with the exception of homologues to OMP P5 of Haemophilus influenzae, were identified. H. influenzae OMP P5 is a major outer membrane protein that is related to the OmpA porin family of proteins [Munson, et al., M Infect Immun, 61:4017-20 (1993)]. OMP P5 in nontypeable Haemophilus influenzae has been shown to encode a fimbrial subunit protein expressed as a filamentous structure [Sirakova, et al., Infect Immun. 62:2002-20 (1994)] that contributes to virulence and binding of both mucin and epithelial cells [Miyamoto and Bakaletz, Microb Pathog. 21:343-56 (1996), Reddy, et al., Infect Immun. 64:1477-9 (1996), Sirakova, et al., Infect Immun. 62:2002-20 (1994)]. A significant finding was identification of two distinct ORF's . that appear to encode OMP P5 homologues. This is also the case with two very similar proteins, MOMP and OmpA2 from Haemophilus ducreyi. It remains to be determined whether both are functionally involved in the production of fimbriae and whether the presence of two such ORFs represents a divergent duplication with redundant or complementing functions. Interestingly, the two OMP P5 mutants seem to have disparate CI values, suggesting a difference in essentiality or functionality for only one copy. OMP P5 has been shown to undergo molecular variation during chronic infections [Duim, et al., Infect Immun. 65:1351-1356 (1997)], however, this

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appears to be restricted to a single gene undergoing point mutations resulting in amino acid changes rather than "type switching" due to differential expression of multiple genes.

Protein folding enzymes are important accessories for the efficient folding of periplasmic and extracellular proteins, and two genes were identified whose products have peptidyl-prolyl isomerase activity: fkpA and tig (trigger factor). FkpA is a periplasmic protein that is a member of the FK506-binding protein family [Horne and Young, Arch Microbiol. 163:357-65 (1995); Missiakas, et al., Mol Microbiol. 21:871-84 (1996)]. FkpA has been shown to contribute to intracellular survival of Salmonella typhimurium [Horne, et al., Infect Immun. 65:806-10 (1997)] and a Legionella pneumophila homolog, mip [Engleberg, et al.; Infect Immun. 57:1263-1270 (1989)], is responsible for virulence and infection of macrophages [Cianciotto, et al., J. Infect. Dis. 162:121-6 (1990); Cianciotto, et al., Infect. Immun. 57:1255-1262 (1989)]. Tig, or trigger factor [Crooke and Wickner, Proc. Natl. Acad. Sci. USA. 84:5216-20 (1987), Guthrie, and Wickner, J Bacteriol. 172:5555-62 (1990), reviewed in Hesterkamp, and Bukau., FEBS Lett. 389:32-4 (1996)], is a peptidyl prolyl isomerase containing a typical FKBP region [Callebaut and Mornon, FEBS Lett. 374:211-215 (1995)], but is unaffected by FK506 [Stoller, et al., EMBO J. 14:4939-48 (1995)]. Tig has been shown to associate with the ribosomes and nascent polypeptide chains [Hesterkamp, et al., Proc Natl Acad Sci USA 93:4437-41 (1996), Stoller, et al., EMBO J. 14:4939-48 (1995)]. Possible roles include an unknown influence on cell division [Guthrie, and Wickner, J Bacteriol, 172:5555-62 (1990)] in E. coli, a role in the secretion and activation of the Streptococcus pyogenes cysteine proteinase [Lyon, et al., EMBO J. 17:6263-75 (1998)] and survival under starvation conditions in Bacillus subtilis [Gothel, et al., Biochemistry 37:13392-9 (1998)].

Bacterial pathogens employ many mechanisms to coordinately regulate gene expression in order to survive a wide variety of environmental conditions within the host. Differences in mRNA stability can modulate gene expression in prokaryotes [Belasco and Higgins, Gene 72:15-23 (1988)]. For example, rnr (vacB) is required for expression of plasmid borne virulence genes in Shigella flexneri [Tobe, et al., J.

Bacteriol. 174:6359-67 (1992)] and encodes the RnaseR ribonuclease [Cheng, et al., J. Biol. Chem. 273:14077-14080 (1998)]. PNP is a polynucleotide phosphorylase that is involved in the degradation of mRNA. Null pnp / rnr mutants are lethal, suggesting a probable overlap of function. It therefore is possible that both rnr and pnp are involved in the regulation of virulence gene expression. A pnp mutant of P. multocida is avirulent in a mouse septicemic model (Example 2)]. Other pnpassociated phenotypes include competence deficiency and cold sensitivity in Bacillus subtilis [Wang and Bechhofer, J Bacteriol. 178:2375-82 (1996)].

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HupA is a bacterial histone-like protein, which in combination with HupB constitute the HU protein in E. coli. Reports have suggested that hupA and hupB single mutants do not demonstrate any observable phenotype [Huisman, et al., J Bacteriol. 171:3704-12 (1989), Wada, et al., J Mol Biol. 204:581-91 (1988)], however, hupA-hupB double mutants have been shown to be cold sensitive, sensitive to heat shock and blocked in many forms of site-specific DNA recombination [Wada, et al., J Mol Biol. 204:581-91 (1988), Wada, et al., Gene. 76:345-52 (1989)]. One limited data previously indicated that hupA is directly involved in virulence [Turner, et al., Infect Immun. 66:2099-106 (1998)]. The mechanism of hupA attenuation remains unknown.

DnaK is a well known and highly conserved heat shock protein

involved in regulatory responses to various stressful environmental changes [reviewed in Lindquist and Craig, Annu Rev Genet. 22:631-77 (1988)]. DnaK is also one of the most significantly induced stress proteins in Yersinia enterocolitica after being phagocytosed by macrophages [Yamamoto, et al., Microbiol Immunol. 38:295-300 (1994)] and a Brucella suis dnaK mutant failed to multiply within human macrophage-like cells [Kohler, et al., Mol Microbiol. 20:701-12 (1996)]. In contrast, another intracellular pathogen, Listeria monocytogenes, did not show induction of dnaK after phagocytosis [Hanawa, et al., Infect Immun. 63:4595-9 (1995)]. A dnaK mutant of Vibrio cholera affected the production of ToxR and its regulated virulence factors in vitro but similar results were not obtained from in vivo grown cells [Chakrabarti, et al., Infect Immun. 67:1025-1033 (1999)]. The Cl of A.

pleuropneumonia dnaK mutant was higher than most of the attenuated mutants although still approximately half of the positive control strain.

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DksA is a dosage dependent suppressor of filamentous and temperature-sensitive growth in a dnaK mutant of E. coli [Kang and Craig, J Bacteriol. 172:2055-64 (1990)]. There is currently no defined molecular function for DksA, but the gene has been identified as being critical for the virulence of Salmonella typhimurium in chickens and newly hatched chicks [Turner, et al., Infect Immun. 66:2099-106 (1998)]. In that work, it was noted that the dksA mutant did not grow well with glucose or histidine but did grow well with glutamine or glutamate as the sole carbon source. This observation may indicate that the dksA mutant is somehow impaired in the biosynthesis of glutamate [Turner, et al., Infect Immun. 66:2099-106 (19981).

Three genes were identified that have roles in protein synthesis: tRNA-leu, tRNA-glu and rpmF. Excluding protein synthesis, tRNA's also have a wide variety of functional roles in peptidoglycan synthesis [Stewart, et al., Nature 230:36-38 (1971)], porphyrin ring synthesis [Jahn, et al., Trends Biochem Sci. 17:215-8 (1992)], targeting of proteins for degradation [Tobias, et al., Science 254:1374-7 (1991)], post-translational addition of amino acids to proteins [Leibowitz and Soffer. B.B.R.C. 36:47-53 (1969)] and mediation of bacterial-eukaryotic interactions [Gray, et al., J Bacteriol, 174:1086-98 (1992), Hromockvi, et al., Mol Microbiol, 6:2113-24 (1992)]. More specifically, tRNA-leu is implicated in transcription attenuation [Carter, et al., Proc. Natl. Acad. Sci. USA 83:8127-8131 (1986)], lesion formation by Pseudomonas syringae [Rich and Willis, J Bacteriol. 179:2247-58 (1997)] and virulence of uropathogenic E. coli [Dobrindt, et al., FEMS Microbiol Lett. 162:135-141 (1998), Ritter, et al., Mol Microbiol. 17:109-21 (1995)]. It is unknown whether the tRNA that we have identified represents a minor species of tRNA-leu in A. pleuropneumoniae. Regardless, it is possible that tRNA-leu may have any one of a wide range of functions. RpmF is a ribosomal protein whose gene is also part of an operon containing fatty acid biosynthesis enzymes in E. coli. Further work will be required to indicate if this is the case in A. pleuropneumoniae, although the same

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clustering of fab genes and rpmF occurs in Haemophilus influenzae [Fleischmann, et al., Science 269:496-512 (1995)]. The expression of the fab genes is not necessarily dependent on transcripts originating upstream of rpmF as there has been a secondary promoter identified within rpmF [Zhang and Cronan, Jr., J Bacteriol. 180:3295-303 (1998)].

The final class of attenuated mutants includes mutations within genes of unknown function or genes that have not been previously identified. Homologs of yaeA and Hl0379 have previously been identified in Escherichia coli [Blattner, et al., Science 277:1453-1474 (1997)] and Haemophilus influenzae [Fleischmann, et al., Science 269:496-512 (1995)], respectively. The remaining unknowns have been designated Actinobacillus pleuropneumoniae virulence genes (apv). The apvC gene shows significant similarity to Hl0893, however, the proposed similarity of Hl0893 as a transcriptional repressor similar to the fatty acid response regulator Bm3R1 [Palmer, J Biol Chem. 273:18109-16 (1998)] is doubtful. The apvD gene is also most similar to a putative membrane protein (b0878) with unknown function from E. coli [Blattner, et al., Science 277:1453-1474 (1997)]. Two other unknowns, apvA and apvB had no significant matches in the public databases.

Example 11 Safety and Efficacy of A. pleuropneumoniae Mutants

Nine groups (n=8) of SPF pigs (4-5 weeks old, 3-10 kg) were used to determine the safety and efficacy of seven *A. pleuropneumoniae* mutants as live attenuated vaccine strains. Seven groups were infected intranasally with 10¹⁰ CFU of each mutant on day 1. One group was vaccinated on days 1 and 15 with the commercially available vaccine Pleuromune (Bayer), and one naive group was not vaccinated. On day 29, all groups were challenged intranasially with 1-5 x 10⁵ CFU per pig of wild type APP225. All surviving animals were euthanized and necropsied on day 42 of the study. Results are shown in Table 4.

Table 4
Efficacy of A. pleuropneumoniae Mutants

	<u>Vaccine</u>	% Mortality following intranasal challenge				
		Vaccination	Challenge			
5	Pleuromune	0	37.5			
	exbB	0	0			
	tig	12.5	0			
	fkpA	12.5	0			
	HI0385	50.0	0			
10	pnp	0	0			
	yaeE	0	0			
	atpG	0	0			
	None	N/A	50.0			

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The exbB, atpG, pnp, and yaeA mutants caused no mortality when administered at a dosage of 10^{10} CFU intranasally. The fkpA and tig mutant groups had one death each and the HI0379 group (highest CI of the 7 mutants tested shown in Example 9) had four deaths. Wildtype LD₅₀ using this model was generally 1×10^7 CFU, indicating that each of these mutants is at least 100 fold attenuated and that there is a reasonable correlation between CI and attenuation.

Example 12 Identification of *P.(Mannheimia) haemolytica* Species Homologs

Based on the sequences of virulence genes identified in P. multocida and A. pleuropneumoniae, attempt were made to identify related genes, i.e., species homologs, in P. (Mannheimia) haemolytica. PCR was utilized with the degenerate primers shown below to attempt amplification of the P. (Mannheimia) haemolytica genes as indicated. Primer sequences, synthesized by Sigma-Genosys (The Woodlands, TX), include standard single letter designations, wherein B indicates

either (C,G or T), D indicates either (G,A or T), H indicates either (A,C or T), K indicates either (G or T), M indicates either (A or C), N indicates either (A,G,C or T), R indicates either (A or G), S indicates either (G or C), V indicates either (G, A, or C), W indicates either (A or T), and Y indicates either (C or T).

	atpG	TEF146 TEF148	ATG GCN GGN GCN AAR GAR AT GCN GCY TTC ATN GCN ACC AT	SEQ ID NO: 176 SEQ ID NO: 177
10	guaB	TEF240 TEF243	GGN TTY ATY CAY AAA AAY ATG TCT TTN GTR ATN GTN ACA TCR TG	SEQ ID NO: 178 SEQ ID NO: 179
	pnp	TEF141 TEF142	GCS GGY AAA CCR CGT TGG GAT TGG CRC CTA ARA TRT CTG AAA GCA CCA C	SEQ ID NO: 180 SEQ ID NO: 181
15	purF	TEF244 TEF247	ATG TGY GGN ATY GTN GGN AT CAT ATC AAT ACC ATA CAC ATT	SEQ ID NO: 182 SEQ ID NO: 183
	yjgF	TEF162 TEF163	GGN CCN TAY GTN CAR G NGC NAC YTC NAC RCA	SEQ ID NO: 184 SEQ ID NO: 185

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For amplification of initial degenerate PCR products, a 50 µl reaction was set up using 3.3X XL buffer II (PE Applied Biosystems), 200 µM dNTPs, 25 pmol each of the appropriate primers, 0.8 mM MgCl₂, 0.5 U rTth DNA polymerase, XL (PE Applied Biosystems) and approximately 1 µg of TF1 DNA.

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Cycle conditions were 94°C for 1.5 min; followed by 35 cycles of 94°C for 15 s, 40-60°C for 60 s, 72°C for 1.5 min; and a final hold at 72°C for 5 min. Each PCR product was band purified from an agarose gel using the QIAGEN Gel Extraction Kit (QIAGEN, Valencia CA).

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Sequencing reactions were performed using the BigDyetm Dye Terminator Chemistry kit from PE Applied Biosystems (Foster City, CA) and run on an ABI Prism 377 DNA Sequencer. Double stranded sequence for the open reading frame (ORF) for each clone was obtained. Sequencher 3.0 software (Genecodes, Corp., Ann Arbor, MI) was used to assemble and analyze sequence data. GCG programs were used to confirm the identity of the ORF by searching for homologous sequences in currently available databases.

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The Vectorette Kit (Genosys Biotechnologies, The Woodlands, TX) was used to obtain additional flanking sequence for each of the genes. Vectorette libraries were prepared according to the manufacturer's suggested protocol. Perkin Elmer Applied Biosystems GeneAmp XL PCR Kit components were used to create the Vectorette PCR products with the following reaction conditions. A 50 µl reaction was set up using 3.3X XL buffer II (PE Applied Biosystems), 200 µM dNTPs, 25 pmol each of the appropriate primers(shown below), 0.8 mM MgCl₃, 0.5 U rTth DNA polymerase, XL (PE Applied Biosystems) and 1 µl of the appropriate vectorette library. Cycle conditions were 94°C for 1.5 min; followed by 35 cycles of 94°C for 20 s, 60°C for 45s, 72°C for 4 min; and a final hold of 72°C for 7 min. The second primer for each library was the manufacturer's vectorette primer.

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Table 5

Gene	Vectorette library	Primer(s)
atpG	BglII, HindIII	TEF217 GAAGCCGCCATACGCTCTTGGG
		SEQ ID NO: 18
	ClaI	TEF218 GTTGCTTCCTTTGCCTGCACTGG
		SEQ ID NO: 18
guaB	EcoRI	TEF265 GGCTCAGAAACAATACCACTTTCA
guan	Lord	SEQ ID NO: 18
-	HindIII, Taq1	TEF268 GCACCAAAGCAGAATTTGTCC
		SEQ ID NO: 18
pnp	ClaI, HincII	TEF219 GGTGATGATGTCGATGATAGTCCC
		SEQ ID NO: 19
	TaqI,	TEF220 GGCGTATTAGCCGTGATGCCAACC
	BamHI	SEQ ID NO: 19 TEF286 GACCACTTAGGCGATATGGACTT
	Damrii	
		SEQ ID NO: 19:
purF	TaqI	TEF271 ACCATCATAAATCGCCTGATTC
		SEQ ID NO: 19:
		TEF292 ACCTGCGGCATCTTGTCCTC SEQ ID NO: 194
	HincII	TEF274 ACGGGTTTATTTTGCCTCTG
		SEQ ID NO: 195
yjgF	ClaI	TEF221 CGCCGGTTTCAGGATTCACGGG
JJ6-		SEO ID NO: 196
	EcorV	TEF281 CTGAACAACGTGAAAGCCAT
		SEQ ID NO: 197

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Vectorette PCR products were band purified and sequenced as described above.

Polynucleotide sequences for the atpG, guaB, pnp, purF, and yjgF genes are set out in SEQ ID NOs: 166, 168, 170, 172 and 174, respectively. Polypeptides encoded by these genes are set out in SEQ ID NOs: 167, 169, 171, 173, and 175, respectively.

Numerous modifications and variations in the invention as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.

WHAT IS CLAIMED IS:

 A gram-negative bacteria comprising a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 7, 9, 21, 25, 27, 29, 39, 41, 51, 53, 55, 57, 58, 60, 68, 72, 74, 76, 78, 80, 82, 84, 104, 108, 112, 116, 118, 120
 122, 124, 126, 128, and 130, or species homologs thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.

- The gram-negative bacteria of claim 1 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.
- The gram-negative bacteria of claim 1 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
- The gram-negative bacteria of claim 1 wherein said mutation results in deletion of all or part of said gene.
- 5. An attenuated *Pasteurellaceae* bacteria comprising a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172 and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.
- The Pasteurellaceae bacteria of claim 5 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.
- The Pasteurellaceae bacteria of claim 5 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.

 The Pasteurellaceae bacteria of claim 5 wherein said mutation results in deletion of all or part of said gene.

- The Pasteurellaceae bacteria of claim 5 selected from the group consisting of Pasteurella (Mannheimia) haemolytica, Pasteurella multocida, Actinobacillus pleuropneumoniae and Haemophilus somnus.
- 10. The Pasteurellaceae bacteria of claim 9 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.
- 11. The Pasteurellaceae bacteria of claim 9 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
- 12. The Pasteurellaceae bacteria of claim 9 wherein said mutation results in deletion of all or part of said gene.
- 13. The attenuated Pasteurellaceae bacteria of claim 9 that is a P. multocida bacteria.
- 14. The Pasteurellaceae bacteria of claim 13 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.
- 15. The Pasteurellaceae bacteria of claim 13 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
- 16. The Pasteurellaceae bacteria of claim 13 wherein said mutation results in deletion of all or part of said gene.
- The attenuated Pasteurellaceae bacteria of claim 9 that is a A. pleuropneumoniae bacteria.

18. The Pasteurellaceae bacteria of claim 17 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.

- 19. The Pasteurellaceae bacteria of claim 17 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
- 20. The Pasteurellaceae bacteria of claim 17 wherein said mutation results in deletion of all or part of said gene.
- An immunogenic composition comprising the bacteria according to any one of claims 1 through 20.
- A vaccine composition comprising the immunogenic composition according to claim 21 and a pharmaceutically acceptable carrier.
- The vaccine composition according to claim. 22 further comprising an adjuvant.
- 24. A method for producing a gram-negative bacteria mutant comprising the step of introducing a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.
- 25. A method for producing an attenuated *Pasteurellaceae* bacteria comprising the step of introducing a mutation in a gene represented by a nucleotide sequence set forth in any one of SEO ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29.

31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172, and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.

- 26. A purified and isolated *Pasteurellaceae* polynucleotide comprising a nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, 164, 166, 168, 170, 172 and 174.
- 27. A purified and isolated *Pasteurellaceae* polynucleotide comprising a nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 21, 25, 27, 29, 39, 41, 51, 53, 55, 57, 58, 60, 68, 72, 74, 76, 78, 80, 82, 84, 104, 108, 112, 116, 118, 120 122, 124, 126, 128, and 130.
- 28. A purified and isolated polynucleotide encoding a Pasteurellaceae. virulence gene product, or species homolog thereof, selected from the group consisting of:
 - a) the polynucleotide according to claim 27,
 - b) polynucleotides encoding a polypeptide encoded by the polynucleotide of (a), and
 - c) polynucleotides that hybridize to the complement of the polynucleotides of (a) or (b) under moderate stringency conditions.
- 29. A purified and isolated *Pasteurellaceae* polynucleotide encoding a polypeptide selected from the group consisting of polypeptides having amino acid sequences set forth in SEQ ID NOs: 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 32, 34, 38, 40, 42, 52, 54, 56, 59, 61, 69, 71, 73, 75, 77, 79, 81, 83, 85, 101, 103, 105, 107, 109,

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- 30. The polynucleotide of claim 29 which is a DNA.
- 31. A vector comprising the DNA of claim 30.
- 32. The vector of claim 31 that is an expression vector, wherein the DNA is operatively linked to an expression control DNA sequence.
- 33. A host cell stably transformed or transfected with the DNA of claim 30 in a manner allowing the expression of the encoded polypeptide in said host cell.
- 34. A method for producing a recombinant polypeptide comprising culturing the host cell of claim 33 in a nutrient medium and isolating the encoded polypeptide from said host cell or said nutrient medium.
 - 35. A purified polypeptide produced by the method of claim 34.
- 36. A purified polypeptide comprising a polypeptide selected from the group consisting of polypeptides having amino acid sequences set forth in SEQ ID NOs: 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 32, 34, 38, 40, 42, 52, 54, 56, 59, 61, 69, 71, 73, 75, 77, 79, 81, 83, 85, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 165, 167, 169, 171, 173, and 175.
- An antibody that is specifically reactive with the polypeptide of claim
 36.
 - 38. The antibody of claim 33 that is a monoclonal antibody.

39. A method of using the monoclonal antibody of claim 39 for identifying a bacteria of claim 1, 5, 9, or 13 comprising the step of contacting an extract of bacteria with said monoclonal antibody and detecting the absence of binding of said monoclonal antibody.

- 40. A method of identifying an anti-bacterial agent comprising the steps of assaying potential agents for the ability to interfere with expression or activity of gene products represented by the amino acid sequences set forth in any one of SEQ ID NOS: 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 32, 34, 38, 40, 42, 52, 54, 56, 59, 61, 69, 71, 73, 75, 77, 79, 81, 83, 85, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 165, 167, 169, 171, 173, and 175 and identifying an agent that interferes with expression or activity of said gene products.
- A method of identifying an anti-bacterial agent comprising the steps of:
 - a) measuring expression or activity of a gene product as set out in SEQ ID NOS: 2, 4, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 32, 34, 38, 40, 42, 52, 54, 56, 59, 61, 69, 71, 73, 75, 77, 79, 81, 83, 85, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 165, 167, 169, 171, 173, and 175:
 - b) contacting the gene product in (a) with a test compound
 - c) measuring expression or activity of the gene product in the presence of the test compound; and
 - d) identifying the test compound as an antibacterial agent when expression or activity of the gene product is decreased in the presence of the test compound as compared to expression or activity in the presence of the test compound.

SEQUENCE LISTING

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4

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aat gto tta gaa goo goo ato caa aac cag ata aaa cgo gto gto tgt 14 Asn Val Leu Glu Ala Ala Ile Gln Asn Gln Ile Lys Arg Val Val Cys 45	4
ctt ag: aca gat aaa gcg gtg tac cca att aat gcg atg ggc att tct 19 : Leu Ser Thr Asp Lys Ala Val Tyr Pro Ile Asn Ala Met Gly Ile Ser 50	2
aaa gca atg atg gaa aaa gtc atc atc gca aaa tcg cgt aac cta gaa 24 Lys Ala Met Met Glu Lys Val Ile Ile Ala Lys Ser Arg Asn Leu Glu 65 70 70 80	0
ggc aca cca acg aca atc tgt tgt act cgc tat ggc act gtc atg gca 28 Gly Thr Pro Thr Thr Ile Cys Cys Thr Arg Tyr Gly Asn Val Met Ala 85 95	8
tog cgt ggt tog gtt atc oca tta ttt gtc gat caa ata cgt caa ggc 336 Ser Arg Gly Ser Val Ile Pro Leu Phe Val Amm Gln Ile Arg Gln Gly 100 105 110	6
and cot ttt act att act gat cot gag atg aca cgc ttt atg atg aca $$ 384 Lys Pro Phe Thr Ile Thr Asp Pro Glu Met Thr Arg Phe Met Met Thr $$ 125 $$ 120 $$ 125	4
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ott gcc aaa gca att acc gaa tta tta tot gtc cca aat cac oct att 526 Leu Ala Lys Ala Ile Thr Glu Leu Leu Ser Val Pro Asn His Pro Ile 165 170 175	8
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7

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Gly Glu Pro Lys Ile Thr Glu Val Thr Asp Tyr Asn Ser His Asn Thr
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Phe Ile Gln Lys Met Ile Glu Gly Glu Tyr Ile Ser Pro Glu Val
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Leu Ser Thr Asp Lys Ala Val Tyr Pro Ile Asn Ala Met Gly Ile Ser
Lys Ala Met Met Glu Lys Val Ile Ile Ala Lys Ser Arg Asn Leu Glu
Gly Thr Pro Thr Thr Ile Cys Cys Thr Arg Tyr Gly Asn Val Met Ala
Ser Arg Gly Ser Val Ile Pro Leu Phe Val Asp Gln Ile Arg Gln Gly
Lys Pro Phe Thr Ile Thr Asp Pro Glu Met Thr Arg Phe Met Met Thr
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Ser Ile Ile Gly Thr Arg His Gly Glu Lys Ala Phe Glu Ala Leu Leu
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Ile Pro Ala Asp Gln Arg Ser Leu Asn Tyr Ser Lys Tyr Val Glu Lys
Gly Glu Pro Lys Ile Thr Glu Val Thr Asp Tyr Asn Ser His Asn Thr
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Pro His Gln Thr Asp Phe Asp Asp Pro His Phe Ala Val Ile Ala Lys

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                                      155
                                                          160
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6873

6876

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32

Ser Tyr Asp Glu Met Arg Asn Lys Trp Lys Ser Phe Lys Glu Asn Pro 260 265 270

Thr Asp Phe Ile Tyr Tyr Pro Ser Glu Lys Ala Lys Ile Leu Ala Gly Lys Leu Glu Gly Lys Leu Thr Thr Leu Gln Asn Gly Glu Tyr Ala Glu Arg Gly Lys Phe Asp Glu Ser Ile Gln Ile Gly Lys His Gln Leu Ser Leu Pro Ser Val Glu Leu Lys Ala Glu Phe Ser Asp Lys Glu Arg Leu Glu Glu Asp Gly Val Asp Leu Ser Ser Ile Ala Glu Leu Leu Glu Met Pro Asn Leu Phe Ile Asp Asn Ser Ile Gln Leu Glu Lys Lys Leu Ser Pro Ile Glu Asp Leu Asp Glu Glu Pro Arg Lys Asn Leu Asp Ile Glu Glu Ser His Ser Asn Ser Ser Asp Asp Val Leu Ser Met Asn Asp Asp Glu Ser Asp Thr Asp Asp Ser Lys Trp Ser Met Gly Asn Asp Glu Lys Glu Met Pro Asp Asp Lys Leu Gly Ile Ser Arg Asp Asp Arg Gly Asn Lys Pro Pro Arg Thr Asp Pro Thr Val Asp Tyr Leu Asn Pro Asp Glu Phe Phe Glu Asn Gly Tyr Leu Leu Asn Glu Leu Leu Gln Glu Leu Gly Glu Glu Pro Leu Leu Lys Glu Gly Glu Asp His Phe Lys Arg Ser Thr Asn Leu Val Arg Leu Gly Glu Arg Asp Arg Gln Asn Arg Glu Lys Arg Glu Lys Glu Gly Tyr Phe Asp Leu Pro Gly Thr Leu Asp Met Lys Leu Gln Glu Leu Phe Glu Lys Arg Lys Gln Lys His Glu Ala Glu Gln Lys Ala Arg Ile Glu Lys Ala Leu Leu Gln Lys Ser Glu Gln Glu Lys Arg Val Glu Glu Arg Lys Gln Glu Glu Lys Arg Gln Ala Gln Asp Lys Ile Ala Lys Gln Val Glu Ile Ala Lys Glu Met Gln Arg Val Glu Glu Ile Arg Gln Arg Glu Lys Gln Leu Ala Ile Gln Leu Gln Glu Glu Glu Lys Lys Gln Gln Glu Glu Lys His Leu Ser Glu Glu Lys Lys Gln 600

Ala Glu Gln Lys Gln Lys Ala Glu Glu Lys Val Ala Gln Glu Arg Leu Asp Ile Glu Gln Gln Lys Ala Tyr Glu Glu Met Ala Lys Arg Glu Ala Glu Ala Ser Lys Asn Val Leu Leu Lys Ala Ile Asp Glu Glu Arg Pro Lys Val Glu Thr Asp Pro Leu Phe Arg Thr Lys Leu Lys Tyr Ile Asn Gln Asp Asp Tyr Ala Gly Ala Asn Tyr Phe Phe Asn Lys Val Gly Leu Asn Thr Lys Gly His Gln Lys Val Asn Val Leu Gly Asp Asn Tyr Phe Asp His Gln Val Ile Thr Arg Ser Ile Glu Lys Lys Val Asp Asn His Leu Asn Gln Lys Tyr Asn Leu Ser Asp Val Glu Leu Val Lys Gln Leu Met Asp Asn Ser Thr Thr Gln Ala Gln Glu Leu Asp Leu Lys Leu Gly Ala Ala Leu Thr Lys Glu Gln Gln Ala Asn Leu Thr Gln Asp Ile Val Trp Tyr Val Lys Thr Lys Val Lys Gly Lys Asp Val Phe Val Pro Lys Val Tyr Phe Ala Ser Glu Thr Leu Val Glu Ala Gln Lys Leu Gln Gly Leu Gly Thr Gly Thr Ile Arg Val Gly Glu Ala Lys Ile Lys Ala Lys Asp Val Val Asn Thr Gly Thr Leu Ala Gly Arg Lys Leu Asn Val Glu Ala Ser Asn Lys Ile Lys Asn Gln Gly Ser Ile Leu Ser Thr Gln Glu Thr Arg Leu Val Gly Arg Lys Gly Ile Glu Asn Val Ser Arg Ser Phe Ala Asn Asp Glu Leu Gly Val Thr Ala Gln Arg Ser Glu Ile Lys Thr Glu Gly His Leu His Leu Glu Thr Asp Lys Asp Ser Thr Ile Asp Val Gln Ala Ser Asp Ile Lys Ala Lys Thr Ser Phe Val Lys Thr Gly Asp 905 Val Asn Leu Lys Asn Thr Tyr Asn Thr Lys His Ala Tyr Arg Glu Lys Phe Ser Pro Ser Ala Leu Gln Val Ala Glu Leu Asp Val Ala Gly Leu 935

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- Thr Ser Glu Ala Thr Ser Glu Gly Ser Ile Phe Glu Val Gly His Leu 965 970 975
- His Leu Ala Val Asp Arg Asp Val Asn Gln Ala Gly Ser Lys Ile Lys 980 985 990
- Ala Lys Tyr Thr Thr Gly Val Val Lys Gly Asn Phe Asn Thr Glu Ala 995 1000 1005
- Gly Lys Asn Ile Lys His Val Glu Lys Glu Glu Tyr Ser Ser Gln Leu 1010 1020
- Phe Ala Ser Ala His Ala Ser Gly Gly Gly Thr Ser Val Arg Tyr Asp 1025 1030 1035 1040
- Tyr Asn Ser Gln Asp Gly Gly Asn Ala Ser Val Gly Val Pro Thr Asn 1045 \$1050
- His Thr Gly Val Gly Ala Glu Ala Gly Met Ser Phe Thr His Thr Lys 1060 \$1050\$
- Asp Lys Glu Thr Leu Leu Thr His Thr Asn Ser Glu Leu Gln Val Lys 1075 1080 1085
- His Gly Lys Leu His Val Leu Gly Tyr Ala Asp Ile Gly Gly Val Asp 1090 $\,$ 1090
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- Gly Asp Leu Ala Gly Ser Ser Ala Lys Leu Ser Val Glu Arg Thr His 1265 1270 1270 1280

35

Glu Thr Lys Arg Thr Thr Glu Thr Gly Asp Ile Val Thr Lys Ile Gly 1285 1290 1295

- Gly Asn Val Thr Leu Ser Ala Arg Ser Gly Ser Val Asn Leu Lys Asn 1300 1305
- Val Gln Ser Asp Glu Gln Ala Asn Leu Thr Leu Arg Ala Lys Glu Asp 1315 1320 1325
- Val Asn Val Leu Ser Gly Glu Lys Thr Arg Glu Thr Thr Glu Thr Val
- Ser Arg Gln Lys Leu Ser His Gly Val Asn Ala Gly Cys Ser Met Met 1345 1355 1360
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- Ala Arg Asn Met Lys Val Glu Ala Gly Arg Asp Phe Asn Val Val Ser 1395
- Ser Asn Ile Asp Ala Asp Lys Leu Asp Leu His Val Lys Gly Lys Thr 1410 1415 1420
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- Lys Arg Thr Val Asn Gln Gln Ala Gly Ile Lys Ala Asn Lys Ile Thr $1475 \ \ \, 1480 \ \ \, 1485$
- Gly Gln Thr His Asp Leu Asn Leu Glu Gly Gly Tyr Leu Val Ser Asn 1490 1500
- Asp Lys Asp Asn Gln Leu Lys Val Thr Gly Asp Val Thr Thr Lys Ala 1505 1510 1515 1520
- Gly Ile Ser Glu Arg Gly Thr Thr Ala Phe Asn Val Arg Gly Gly Arg 1540 1545
- Ala Glu Gln Lys His Tyr Asn Ala Thr Gln Lys Ser Thr Leu Ser Gly 1555 \$1560\$
- Val Asp Thr Ser Gln Ala Asn Val Ser Gly Gln Val Asn Thr Asp Leu 1570 1575 1580
- Thr Lys Ala Lys Ala Val Thr Arg Asp Asp Thr Tyr Ala Ser Thr Gln 1585 1590 1595
- Phe Ser Phe Glu Val Ala Asp Ile Val Glu Leu Gly Gln Arg Ala Lys 1615 1615

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Thr Leu Arg Ser Arg Ser Thr Thr Glu Glu Ala Asp Val Pro Thr Thr 1635

Arg Ser Arg Val Thr Asp Glu Ala Asp Ser Val Ser Val Lys Asn Pro 1650 1660

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Val Asp Ser Thr Asp Leu Val Asp Asn Pro Leu Tyr Ala Ser Ala Thr 1695

Thr Lys Ala Asn Ile His Asp Tyr Glu Glu Ile Pro Ala Val Tyr Ser 1700 1705 1710

Lys Val Gly Asp Asn Asn Ala Asp Leu Val Arg His Lys Thr Ala Thr 1715 1720 1725

Ser Asp Glu His Leu Tyr Ala Glu Ile Asn Glu Pro Thr Tyr Ser Arg 1730 1730 1740

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Thr Asp Tyr Ala Asp Val Val Gln Ala His Thr Arg Lys Ala Asp Asp 1765 1770 1770 1775

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Gly Ser Glu His Ile Tyr Thr Asp Ile Ser Asp Val Gly Thr Gln Thr 1795 1800 1805

Lys Ala Ile Asp Ser Thr Tyr Ala Thr Val Gly Met Pro Lys Ala Asn 1810 1815 1820

Ala Val Asn Leu Ile Gly Gln Asn Gly Leu Gly Ser Ile Tyr His Ser 1825 1830 1835

Pro Asp Ser Ala Tyr Lys Thr Trp Gln Leu Leu Asp Gln Phe Ala Asn 1845 1850 1855

Lys Gly Gly Asp Ala Val Phe Leu Arg Pro Ala Thr Glu Met Lys Cys 1860 1865 1870

Ala Gly Ala Pro Leu Lys Tyr Thr Phe Ile Val Arg Asp Tyr Leu Leu 1875 1880

Arg Arg His Thr Leu Asp Lys Ser Arg Leu Phe Tyr Asn Ala His Asn 1890 1895 1900

Lys Thr.Leu Phe Ser Val Pro Ile Val Asp Ala Lys Val Lys Met Leu 1905 $$ 1910 $$ 1915 $$ 1915 $$ 1920 $$

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Ile Asp Leu Ser Lys Arg Ile Ala Thr Phe Asn Ser Pro Glu Gly Val

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                                          1995
Arg Tyr Ala Asn Val Phe Ala Val Gly Asp Val Ala Gly Val Pro Lys
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                                     2010
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His Leu Leu Ala Glu Leu Glu Gly Lys Pro Cys Asp Glu Ile Tyr Asn
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Gly Tyr Thr Ser Cys Pro Leu Ile Thr Gln Leu Gly Lys Gly Met Leu
Val Glu Phe Asp Tyr Asn Asn His Leu Thr Pro Ser Phe Pro Gly Val
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			tta Leu													2072
			cct Pro													2120
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2364

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Tyr Ala Lys Phe Gly Glu Ala Tyr Lys Lys Trp Lys Glu Tyr Leu Pro 650 Lys Asn Ala Glu Glu Asn Ile Ala Tyr Ile Ala Gln Asp Lys Thr Phe Lys Lys His Ser Tyr Ser Leu Gly Ala Thr Phe Asp Pro Leu Asn Phe Leu Arg Val Gln Val Lys Tyr Ser Lys Gly Phe Arg Ala Pro Thr Ser Asp Glu Leu Tyr Phe Thr Phe Lys His Pro Asp Phe Thr Ile Leu Pro Asn Pro Val Leu Lys Pro Glu Glu Ala Lys Asn Gln Glu Ile Ala Leu Thr Val His Asp Asn Trp Gly Phe Val Ser Thr Ser Val Phe Gln Thr Lys Tyr Arg His Phe Ile Asp Leu Ala Tyr Leu Gly Ser Arg Asn Leu Ser Asn Ser Val Gly Gly Gln Ala Gln Ala Arg Asp Phe Gln Val Tyr Gln Asn Val Asn Val Asp Asn Ala Lys Val Lys Gly Leu Glu Ile Asn 785 790 795 800 Ala Arg Leu Asn Leu Gly Tyr Phe Trp His Val Leu Asp Gly Phe Asn Thr Ser Tyr Lys Phe Thr Tyr Gln Arg Gly Arg Leu Asp Gly Asp Arg Pro Met Asn Ala Ile Gln Pro Lys Ala Ser Val Phe Gly Leu Gly Tyr Asp His Lys Glu Asn Lys Phe Gly Ala Asp Leu Tyr Ile Thr Arg Val Ser Glu Lys Lys Ala Lys Asp Thr Tyr Asn Met Phe Tyr Lys Glu Gln Gly Tyr Lys Asp Ser Ala Val Arg Trp Arg Ser Asp Asp Tyr Thr Leu Val Asp Ala Val Gly Tyr Ile Lys Pro Ile Lys Asn Leu Thr Leu Gln Phe Gly Val Tyr Asn Leu Thr Asp Arg Lys Tyr Leu Thr Trp Glu Ser Ala Arg Ser Ile Lys Pro Phe Gly Thr Ser Asn Leu Ile Asn Gln Lys 935 Thr Gly Ala Gly Ile Asn Arg Phe Tyr Ser Pro Gly Arg Asn Phe Lys Leu Ser Ala Glu Ile Thr Phe 965

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Tyr Phe Leu Trp Phe Ile Leu Phe Ile Leu Ser Ile Tyr Leu Phe Ile
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acc ata caa qaa aqa cga ggt tat tgt ttt gac aaa cgt gca tat att
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aaa gaa gta aag cgt Lys Glu Val Lys Arg 75					1372
cca gct tgt tgt aca Pro Ala Cys Cys Thi 90	tta acc acc Leu Thr Thr : 95	ttt att gat Phe Ile Asp 100	gaa gga ggo Glu Gly Gly	gat ggc : Asp Gly 105	1420
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cat tat gtt gag aat His Tyr Val Glu Asr 125	Leu Lys Pro'	tat cat aga Tyr His Arg 130	gtg att tat Val Ile Tyr 135	Leu Glu	1516
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Gly Val Ile Lys Pro Asp Gly Thr Ile Lys Glu Val Lys Arg Tyr Thr
Ser Val Glu Glu Phe Lys Gln Met Asn Pro Ala Cys Cys Thr Leu Thr
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gcagaactgg ctagcttatc acttttagat aattgtatta ttaaaaqaaq ctgtatqatt 180
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Asn Glu Lys Gly Ile Ser Phe Tyr Ile Phe Asp Leu Gly Ile Lys Asp
gaa aat aag aga aat att aat gat att gtt tot tot tat gga agt gaa
                                                                     495
Glu Asn Lys Arg Asn Ile Asn Asp Ile Val Ser Ser Tyr Gly Ser Glu
gtc aac ttt att gct gtg aat gag aaa gaa ttt gag agt ttt cct gtt
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Val Asn Phe Ile Ala Val Asn Glu Lys Glu Phe Glu Ser Phe Pro Val
caa att agt tat att tot tta goa aca tat goa agg ota aaa gog goa
                                                                     591
Gln Ile Ser Tyr Ile Ser Leu Ala Thr Tyr Ala Arg Leu Lys Ala Ala
gag tat ttg ccg gat aat tta aat aaa att att tat tta gat gtt gat
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Glu Tyr Leu Pro Asp Asn Leu Asn Lys Ile Ile Tyr Leu Asp Val Asp
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tot gag cat aaa aaa tog att toa atg toa gat aag gaa tat tat ttt 78: Ser Glu His Lys Lys Ser Ile 5er Met Ser Asp Lys Glu Tyr Tyr Phe 140 145 150	3
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Lys Xaa Ser Asn Leu His Ser Leu Glu Lys Thr Thr Met Pro Val Val
225
Ile Ser His Tyr Cys Gly Pro Glu Lys Ala Trp His Ala Asp Cys Lys
His Phe Asn Val Tyr Phe Tyr Gln Lys Ile Leu Ala Xaa Xaa Ser Arg
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Gly His Pro Asp Ala Glu Ala Arg Thr Lys Phe Val Ile Lys Glu Leu
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Ser Lys Ala Asn Gln Ile Glu Val Ile Ile Ala Asn Asn Asp Gly Met
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Ala Met Gly Ala Leu Glu Ala Thr Lys Ala His Gly Lys Lys Leu Pro
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Ile Phe Xaa Val Xaa Ala Leu Pro Glu Val Leu Gln Leu Ile Lys Lys
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Ser Lys Ala Asn Gln Ile Glu Val Ile Ile Ala Asn Asn Asp Gly Met
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75 80		85	
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Val Glu Gln Leu Leu Leu Ser Ly 110			
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98

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	tct Ser	gct Ala 130	gca Ala	cca Pro	gaa Glu	att Ile	cgt Arg 135	tat Tyr	cca Pro	aat Asn	gtg Val	tat Tyr 140	ggt Gly	att Ile	gat Asp	atg Met	433
pana			aaa Lys														481
			tta Leu														529
			ggt Gly														577
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Ile Ile Phe Arg Asp Val Ile Glu Arg Tyr Gln Asn Glu Val Ser Ile
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104

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Thr Lys Gly Tyr Leu Ile Gly Gly Lys Ala Trp Leu Asn Lys Glu Leu Asn Ser Ala Thr Asn Leu Lys Asp Ala Lys Leu Leu Ile Ser Tyr Asp Tyr His Pro Val Pro Pro Leu Glu Gln Leu Gly Phe Asn Gln Tyr Ile Ser Asp Glu Tyr Leu Val Asp Phe Ser Asn Arg Leu Ala Ser <210> 51 <211> 353 <212> DNA <213> Pasteurella multocida <220> <221> CDS <222> (1)..(351) <220× <223> unknown C1 <400> 51 atg aca tta ttt gat gaa tgt aaa tta gct ctt aga gac gat ttt aat Met Thr Leu Phe Asp Glu Cys Lys Leu Ala Leu Arg Asp Asp Phe Asn cta att tgt gat gaa gag aag gat tgt gta atg gat aag ttt tat ttc Leu Ile Cys Asp Glu Glu Lys Asp Cys Val Met Asp Lys Phe Tyr Phe tat ttc ttg gaa aag aaa gag gaa ttt aat ttt caa gat tat tca ttt Tyr Phe Leu Glu Lys Lys Glu Glu Phe Asn Phe Gln Asp Tyr Ser Phe 40 gaa gaa atg tat ata ttt tca aaa atg gaa cct gtg tat gtt tta tgt Glu Glu Met Tyr Ile Phe Ser Lys Met Glu Pro Val Tyr Val Leu Cys gat ago tot aat ata cot tig tit agg agt aat igg gaa tig att ato Asp Ser Ser Asn Ile Pro Leu Phe Arg Ser Asn Trp Glu Leu Ile Ile 65 aat aat ata tat gat gtt gtc tgt tta tct aca aaa gta ttt ttt cta 288 Asn Asn Ile Tyr Asp Val Val Cys Leu Ser Thr Lys Val Phe Phe Leu gat gat gaa aag tta atg atg gaa tta ttt cct gaa gat aaa gta aga Asp Asp Glu Lys Leu Met Met Glu Leu Phe Pro Glu Asp Lys Val Arg 100 105 353 gtc atc tat aaa aga ta Val Ile Tyr Lys Arq 115

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Tyr Glu His Val Tyr Ser Phe Gly Ser Thr Gly Glu Gly His Phe Ile
            100
tgt ttt gat tat cgt gat gat cca aaa ggt gat gaa ccc aaa atc tgt
                                                                      384
Cys Phe Asp Tyr Arg Asp Asp Pro Lys Gly Asp Glu Pro Lys Ile Cys
atc gtg att cac gat gaa tat gat gaa aaa aca ggg aaa atg cga ctg
Ile Val Ile His Asp Glu Tyr Asp Glu Lys Thr Gly Lys Met Arg Leu
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Phe Pro Ile Ala Glu Asn Phe Glu Ala Phe Leu Asp Ser Leu Lys Ser
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Glu Glu Asn Cys Phe Glu Tyr Tyr Asn Glu Arg Asn Glu Pro Thr Phe
Ser Ser Phe Gly Phe Glu Gly Phe Glu Thr Glu Arg Ser Ser Ala Ser
Leu Glu Asn Ile Tyr Ala Gln Tyr Ile Tyr Asp Asp Pro Ile Tyr Gly
Tyr Glu His Val Tyr Ser Phe Gly Ser Thr Gly Glu Gly His Phe Ile
Cys Phe Asp Tyr Arg Asp Asp Pro Lys Gly Asp Glu Pro Lys Ile Cys
Ile Val Ile His Asp Glu Tyr Asp Glu Lys Thr Gly Lys Met Arg Leu
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Gly Lys Asn Glu Ser Asn Lys Asp Ile Leu Lys Leu Val Glu Ile Val
tot toa gat tit gaa gig gat gaa ota agt oat aaa gat gaa oac gag
                                                                   144
Ser Ser Asp Phe Glu Val Asp Glu Leu Ser His Lys Asp Glu His Glu
ata tat tat ttg ttt tat aag agg ggt gtt gaa ttt tgt ttt aaa aga
                                                                   192
Ile Tyr Tyr Leu Phe Tyr Lys Arg Gly Val Glu Phe Cys Phe Lys Arg
ata gat gaa gag tat gto tta tat tog gtt tto ttt tto ttg gta gag
                                                                   240
Ile Asp Glu Glu Tyr Val Leu Tyr Ser Val Phe Phe Leu Val Glu
gtt gat aat tat ttt tca tgc cca ttt att cat gaa tta ata tgt gat
                                                                   288
Val Asp Asn Tyr Phe Ser Cys Pro Phe Ile His Glu Leu Ile Cys Asp
ctt aaa cac gga ttc tca ata gag gat att ata agg ttt tta ggg gag
Leu Lys His Gly Phe Ser Ile Glu Asp Ile Ile Arg Phe Leu Gly Glu
cca aat ttt aaa ggt agt ggc tgg gta aga tat tct tat aat gga aga
Pro Asn Phe Lys Gly Ser Gly Trp Val Arg Tyr Ser Tyr Asn Gly Arg
                                                                   384
aat att cat ttc gaa ttt aat gaa tct aat gaa tta tcc cag att agc
                                                                   432
Asn Ile His Phe Glu Phe Asn Glu Ser Asn Glu Leu Ser Gln Ile Ser
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att ttt att ta
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Ile Phe Ile
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Ile Asn Lys Ala Arg Glu Phe Phe Ile Asn Asp Asn Val Ala Ile Asn
                                    170
Asn Leu Ala Met Leu Asn Ile Ile Asn Gly Asp Phe Asn Asn Ala Val
Ser Leu Leu Pro Gln Tyr Leu Asn Gly Val Lys Asn Ser Arq Leu
                            200
Ile His Asn Leu Val Phe Ala Leu Val Lys Asn Gly Asp Leu Asp Tyr
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Ala Lys Asp Ile Ile Val Lys Glu Arg Leu Asn Thr Ser Pro Asp Asp
225
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Arq
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Lys Asp Asp Thr Ser Phe Val Thr Glu Gly Asn Asn Phe Ile Thr Ala
                                 25
aaa gac aac tta gaa atc acg gca aaa aat qtt caa att qat caa qcq
                                                                  144
Lys Asp Asn Leu Glu Ile Thr Ala Lys Asn Val Gln Ile Asp Gln Ala
aaa aat att caa tta aac gcg aat atc acg atc aat acc aag tct ggt
                                                                  192
Lys Asn Ile Gln Leu Asn Ala Asn Ile Thr Ile Asn Thr Lys Ser Gly
                         55
ttt qtq aat tac qqt acc tta qca aqt qct caa aat tta acq att aat
Phe Val Asn Tyr Gly Thr Leu Ala Ser Ala Gln Asn Leu Thr Ile Asn
65
acc gaa caa ggc agc att tat aac ata ggc ggt atc ttg ggg gcg ggt
Thr Glu Gln Gly Ser Ile Tyr Asn Ile Gly Gly Ile Leu Gly Ala Gly
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132

8	5 90	95	
		gaa aac caa gga gga tat 3 Glu Asn Gln Gly Gly Tyr 110	36
ctt att aat caa gg Leu Ile Asn Gln Gl 115	t aag agt cta ctc cat y Lys Ser Leu Leu His 120	tct gaa ggc gcc atg aac 3 Ser Glu Gly Ala Met Asn 125	84
ctc aca gcg gat cg Leu Thr Ala Asp Ar 130	c acg gty tac aat tta g Thr Val Tyr Asn Leu 135	ggg aat att ttt gct aaa 4 Gly Asn Ile Phe Ala Lys 140	32
ggt gac gcg acg at Gly Asp Ala Thr Il 145	c aat gca aac gcg tta e Asn Ala Asn Ala Leu 150	att aat gat gtt act ctc 4 Ile Asn Asp Val Thr Leu 155 160	80
aca ggt cgt ctt ga Thr Gly Arg Leu Gl 16	u Tyr Gln Asp Leu Lys	aaa gat tat acg cgt tat 5 Lys Asp Tyr Thr Arg Tyr 175	28
tat cgt atc aat ga Tyr Arg Ile Asn Gl 180	a acg gca aaa cat ggt u Thr Ala Lys His Gly 185	tgg cat aat aac ttc tat 5 Trp His Asn Asn Phe Tyr 190	76
gaa tta aac gtc ga Glu Leu Asn Val As 195	e aga gtt tct tgatttgt p Arg Val Ser 200	gc atcaattttg taaccaccgg 6	30
ttaataaaac accagca	att tcaacgccat tcatggc	aga taatgccgct gcgacgatca 6	90
catcaggacg atccgcg	gaa gtgacaagta aacttcc	aac goggaaatgt tocaccatat 7	50
tggtcaaatt acgtgca	ag aaagtgatge cacgaat	gcg acgttcattg atcgcgcctt 8	10
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ctgcgctcca aggaata	cat gccaagattt taattgg	gct tttctcaaat aaatgataaa 9	30
tctcagatac ttgattt	gt gtgtgttgga aagaatc	aaa aatttetgee aagteaggge 99	90
gagtacgacc agattca	ca atcggcgcat taaattt	att gatcacaaca ccaagtaaat 10	050
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		cgt agggattaaa ccttccacca 12	
		ttc aacaattttt tctagtacca 17	
		act taacataaat ggttcactgg 13	
		gcg atcaatcata tetteacetg 14	
		cgc ccctttttgc tccagtgcat 14	
		agc actaatcggg ataaggataa 15	
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yaarcggcag ttaattga	te tetacgegat geaaagg	cgc gcggtatctt gtgcaataac 16	550

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Lys Asp Asn Leu Glu Ile Thr Ala Lys Asn Val Gln Ile Asp Gln Ala
Lys Asn Ile Gln Leu Asn Ala Asn Ile Thr Ile Asn Thr Lys Ser Gly
Phe Val Asn Tyr Gly Thr Leu Ala Ser Ala Gln Asn Leu Thr Ile Asn
Thr Glu Gln Gly Ser Ile Tyr Asn Ile Gly Gly Ile Leu Gly Ala Gly
Lys Ser Leu Asn Leu Ser Ala Lys Arg Gly Glu Asn Gln Gly Gly Tyr
Leu Ile Asn Gln Gly Lys Ser Leu Leu His Ser Glu Gly Ala Met Asn
Leu Thr Ala Asp Arg Thr Val Tyr Asn Leu Gly Asn Ile Phe Ala Lys
Gly Asp Ala Thr Ile Asn Ala Asn Ala Leu Ile Asn Asp Val Thr Leu
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Tyr Arg Ile Asn Glu Thr Ala Lys His Gly Trp His Asn Asn Phe Tyr
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                                                       Met Lys Ile
act att aca cga aat cat cca gaa gta ttt caa gaa tcc gct cgt tta
Thr Ile Thr Arg Asn His Pro Glu Val Phe Gln Glu Ser Ala Arg Leu
gta gcc gaa aag ttc att aaa gcc caa tgt gta gaa gca tta aca ttg
                                                                       212
Val Ala Glu Lys Phe Ile Lys Ala Gln Cys Val Glu Ala Leu Thr Leu
                      25
gct ttg att gag ggt gtc gag cac ttt gtg ctg gaa ggt gag gaa gla Ala Leu Ile Glu Gly Val Glu His Phe Val Leu Glu Gly Glu Glu Glu
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agc aaa agg gga cat agt
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Ser Lys Arg Gly His Ser
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Ala Arg Leu Val Ala Glu Lys Phe Ile Lys Ala Gln Cys Val Glu Ala
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gca atg cgt gca tat ctt gat aaa gaa cag ggc tgg cat acg tct att
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Ala Met Arg λ la Tyr Leu Asp Lys Glu Gln Gly Trp His Thr Ser Ile 20 30	
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ttt gåc att aac gac age teg aet gåt gtg aac tat etc aat gaa caa Phe Asp Ile Asn Asp Ser Ser Thr Asp Val Asn Tyr Leu Asn Glu Gln 50 60	192
ggc atc acg tgt tgc gtg aat cat aat ggc ttt cgt ttt tgg ggc tta Gly Ile Thr Cys Cys Val Asn His Asn Gly Phe Arg Phe Trp Gly Leu 65 70 80	240
cgc acg act gca gaa gat cca tta ttc aag ttt gaa gtg tac acc cgc Arg Thr Thr Ale Glu Asp Pro Leu Phe Lys Phe Glu Val Tyr Thr Arg 99 95	288
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gtg gat aaa gat att tot gtc acg cta gtg aaa gat att att gaa gca Val Asp Lys Asp Ile Ser Val Thr Leu Val Lys Asp Ile Ile Glu Ala 120	384
atc aat gcg aag tgg cgt gat tac acc aca aaa ggc tac tta att ggc Ile Aan Ala Lys Trp Arg Asp Tyr Thr Thr Lys Gly Tyr Leu Ile Gly 130	432
ggt aaa gcg tgg ctt aat aaa gag ctt aac agt gca acg aat tta aa Gly Lys Ala Trp Leu Asn Lys Glu Leu Asn Ser Ala Thr Asn Leu Lys 145 150	480
gat gcg aag ttg ttg atc tct tat gat tat cac cca gta cca ccg ctc Asp Ala Lys Leu Leu Ile Ser Tyr Asp Tyr His Pro Val Pro Pro Leu 165 170 175 175 175 175 175 175 175 175 175 175	528
gaa Cag cta ggc ttt aat cag tac att tct gat gaa tac ctt gtt gat Glu Gln Leu Gly Phe Asn Gln Tyr Ile Ser Asp Glu Tyr Leu Val Asp 180 185 190 190 195 190 195 190 195 190 195 190 195 195 195 195 195 195 195 195 195 195	576
ttt tca aat ogt tta gca tcg taaggggtag aaaatggctt taccacgcaa Phe Ser Aen Arg Leu Ala Ser 195	627
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gactcaacca aaattagcaa tgaaaatcga agaatttegc gegggeggta tgattggtte	747
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Phe Asp Ile Asn Asp Ser Ser Thr Asp Val Asn Tyr Leu Asn Glu Gln
Gly Ile Thr Cys Cys Val Asn His Asn Gly Phe Arg Phe Trp Gly Leu
Arg Thr Thr Ala Glu Asp Pro Leu Phe Lys Phe Glu Val Tyr Thr Arg
Thr Ala Gln Ile Leu Lys Asp Thr Ile Ala Gly Ala Phe Asp Trp Ala
Val Asp Lys Asp Ile Ser Val Thr Leu Val Lys Asp Ile Ile Glu Ala
Ile Asn Ala Lys Trp Arg Asp Tyr Thr Thr Lys Gly Tyr Leu Ile Gly
Gly Lys Ala Trp Leu Asn Lys Glu Leu Asn Ser Ala Thr Asn Leu Lys
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										tgg Trp 165						1551
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ttg Leu	tgg Trp	gga Gly	gga Gly 190	cgt Arg	gaa Glu	gga Gly	tat Tyr	gaa Glu 195	acg Thr	tta Leu	tta Leu	aat Asn	acc Thr 200	aat Asn	tta Leu	1647
										atg Met						1695
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										tat Tyr 245						1791
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										cac His						1887
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2584

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200

195

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Phe Gly Leu Glu Lys Glu Ile Lys Val Asn Ile Glu Ala Asn His Ala
Thr Leu Ala Gly His Thr Phe Gln His Glu Val Ala Met Ala Thr Ala
Leu Asp Ile Phe Gly Ser Ile Asp Ala Asn Arg Gly Asp Pro Gln Leu
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Val Ile Tyr Glu Ile Leu Lys Ala Gly Gly Phe Thr Thr Gly Gly Phe
Asn Phe Asp Ala Lys Ile Arg Arg Gln Ser Thr Asp Pro Tyr Asp Leu
Phe His Gly His Ile Gly Ala Ile Asp Val Leu Ala Leu Ser Leu Lys
Cys Ala Ala Lys Met Leu Glu Glu Gln Ala Leu Gln Lys Val Val Asn
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								gtg Val								489
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Leu Tyr Gln Gln Leu Gly His Tyr Arg Ser Gln Glu Ala Ala Val Thr
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	gat Asp 70															1795
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	act Thr 150															2035
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149

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Glu Gln Ser Ala Met Ala Lys Gln Pro Asn Ser Leu Ile Arg Leu Ile
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Pro Ser Lys Ala Ile Ile Ser Ala Pro Ala Ala Ala Asn Ser Ser Met
Ser Cys Lys Asn Gly Leu Ile Arg Thr Gly Leu Ser Gly Lys Ser Arg
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Ser Val Phe Ile Leu Ala Cys Phe Phe Tyr Tyr Arg Ala Glu Leu Thr
Ser Ser Gly Ala Gly Val Gln Ser Val Ala Met Leu Pro Ser Ser Ser
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Val Leu Tyr Ala Phe Lys Asn Gly Gln Asn Gly Asp Val Phe Val Gln
Lys Ala Pro Ala Ala Thr Ile Gly Thr Leu Ala Lys Ala Ile Thr Glu
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Gly Glu Lys Ala Phe Glu Ala Leu Leu Ser Arg Glu Glu Met Val His
                                265
Ala Ile Asn Glu Gly Asn Tyr Tyr Arg Ile Pro Ala Asp Gln Arg Ser
Leu Asn Tyr Ser Lys Tyr Val Glu Lys Gly Glu Pro Lys Ile Thr Glu
Val Thr Asp Tyr Asn Ser His Asn Thr Glu Arg Leu Thr Val Lys Glu
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ctc gtt cct gtg gca gaa tgt att aac tca gct att agc aat ggt tca
Leu Val Pro Val Ala Glu Cys Ile Asn Ser Ala Ile Ser Asn Gly Ser
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gat gga tta aag tat tta gat att att gct aaa aaa att gaa caa aag

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			ggt Gly													960
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tct Ser	agt Ser	aca Thr	ggc Gly 340	gca Ala	atg Met	cat His	ggt Gly	aaa Lys 345	aat Asn	att Ile	aag Lys	t tg Leu	att Ile 350	gtg Val	aca Thr	1056
gat Asp	aaa Lys	ggt Gly 355	gca Ala	ggc Gly	gta Val	aaa Lys	cat His 360	gat Asp	gga .Gly	att Ile	att Ile	ttg Leu 365	tct Ser	gaa Glu	aat Asn	1104
gat Asp	att Ile 370	cag Gln	att Ile	gaa Glu	atg Met	aat Asn 375	gaa Glu	ggt Gly	gac Asp	tta Leu	gaa Glu 380	ctt Leu	ggc Gly	aat Asn	acg Thr	1152
att Ile 385	Cag Gln	caa Gln	aca Thr	gtg Val	gta Val 390	aaa Lys	aaa Lys	gac Asp	cga Arg	aat Asn 395	att Ile	cga Arg	gcc Ala	aag Lys	aaa Lys 400	1200
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Lys	Ser	Asp	gaa Glu 420	Ile	Ser	Leu	Glu	Ala 425	Lys	Gln	Val	Lys	11e 430	Arg	Lys	1296
Asn	Ala	Glu 435	att Ile	Arg	Ser	Thr	Thr 440	Gln	Ala	Lys	Ile	Val 445	Ala	Lys	Gly	1344
Ala	Leu 450	Ser	att Ile	Glu	Gln	Asn 455	Ala	Lys	Leu	Val	Ala 460	Lys	Lys	Ile	Asp	1392
Val 465	Ala	Thr	gaa Glu	Thr	Leu 470	Thr	Asn	Ala	Gly	Arg 475	Ile	Tyr	Gly	Arg	Glu 480	1440
Val	Lys	Leu	gac Asp	Thr 485	Asn	Asn	Leu	Ile	Asn 490	Asp	Lys	Glu	Ile	Tyr 495	Ala	1488
Glu	Arg	Lys	ttg Leu 500	Ser	Ile	Leu	Thr	Lys 505	Gly	Lys	Asp	Leu	Glu 510	Ile	Ile	1536
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gcc Ala	caa Gln	aat Asn 595	att Ile	g aa Glu	att Ile	gat Asp	ааа Lув 600	aat Asn	caa Gln	gat Asp	att Ile	caa Gln 605	ttg Leu	ggt Gly	gct Ala	1824
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gct Ala	aaa Lys	tca Ser	acg Thr 660	gaa Glu	gaa Glu	ggt Gly	atg Met	gga Gly 665	aat Asn	att Ile	gtt Val	aac Asn	caa Gln 670	gaa Glu	aac Asn	2016
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	ccg Pro															2400
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aat Asn	gca Ala	tta Leu	gca Ala 820	agc Ser	gtg Val	ttt Phe	aag Lys	aat Asn 825	cca Pro	gcg Ala	aaa Lys	atc Ile	acg Thr 830	atg Met	tac Tyr	2496
tat Tyr	caa Gln	cca Pro 835	ctt Leu	act Thr	cgt Arg	tat Tyr	att Ile 840	tgg Trp	aca Thr	cca Pro	tta Leu	tcg Ser 845	ggt Gly	aat Asn	gca Ala	2544
tcg Ser	cgt Arg 850	gaa Glu	ttt Phe	aac Asn	aat Asn	tta Leu 855	gag Glu	tct Ser	ttc Phe	ctc Leu	gat Asp 860	gcc Ala	ttg Leu	ttt Phe	ggc Gly	2592
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gag Glu 102!	Asp	cta Leu	gat Asp	gaa Glu	ga G1 103	u Pr	a cg	t aa g Ly	a aars As	n Le	g g u A 135	at a	ita g	gaa g Glu (gaa ag Slu Se 10	r

cat tot aat toa tog gat gac gtg ott agc atg aat gat gat gag tot 3 His Ser Asn Ser Ser Asp Asp Val Leu Ser Met Asn Asp Asp Glu Ser 1045	168
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ccc gat gat aag ctg ggt ata agt cgt gat gat cgt gga aat aaa cca 3: Pro Aap Asp Lys Leu Gly Ile Ser Arg Asp Asp Arg Gly Asn Lys Pro 1075 1080	264
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gaa aat ggt tat ctc ttg aat gag cta cta cag gag ctt gga gaa gag 3: Glu Asn Gly Tyr Leu Leu Asn Glu Leu Leu Gln Glu Leu Gly Glu Clu 1110 1115 1110	360
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Lys Asn Val Leu 1300	aaa gcg att gat gaa Lys Ala Ile Asp Glu 1305	Glu Arg Pro Lys Val	Ğlu
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ggt cat caa aaa gta Gly His Gln Lys Val 1345	aat gtg tta ggg gat Asn Val Leu Gly Asp 1350	aac tat ttt gat cat Asn Tyr Phe Asp His 1355	caa 4080 Gln 1360
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Val Gly Arg Lys Gly 1505	Ile Glu Asn Val Ser	Arg Ser Phe Ala Asn 1515	Asp

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Ser Asp Ser Thr Ser Thr Ser Glu Glu Val Glu Glu Pro Phe Leu	
35 40 45	
35 40 45 Leu Glu Gln Tyr Ser Leu Ser Ser Val Ser Leu Leu Val Lys Ser Thr 50 60	
Leu Glu Gln Tyr Ser Leu Ser Ser Val Ser Leu Leu Val Lys Ser Thr	
Leu Glu Gln Tyr Ser Leu Ser Ser Val Ser Leu Leu Val Lys Ser Thr 50 60 Phe Asn Pro Val Ser Tyr Ala Met Gln Leu Thr Trp Lys Gln Leu Ser	
Leu Glu Gln Tyr Ser Leu Ser Ser Val Ser Leu Leu Val Lys Ser Thr 50 Phe Asn Pro Val Ser Tyr Ala Met Gln Leu Thr Trp Lys Gln Leu Ser 75 To 80 Ile Leu Phe Leu Thr Val Ile Ser Val Pro Val Leu Ala Glu Gly Lys	
Leu Glu Gln Tyr Ser Leu Ser Ser Val Ser Leu Leu Val Lys Ser Thr 50 Phe Asn Pro Val Ser Tyr Ala Met Gln Leu Thr Trp Lys Gln Leu Ser 75 Ro Ile Leu Phe Leu Thr Val Ile Ser Val Pro Val Leu Ala Glu Gly Lys 85 Gly Asp Glu Arg Asn Gln Leu Thr Val Ile Asp Asn Ser Asp His Ile	

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Ala	Val	Phe	Asn	Asn 165	Asn	Gly	Thr	Glu	Ala 170	Gln	Ala	Arg	Ser	Thr 175	Leu
Ile	Gly	Tyr	Ile 180	Pro	Gln	Asn	Gln	Asn 185	Leu	Arg	Gly	Gly	Lys 190	Glu	Ala
Asp	Val	Ile 195	Leu	Asn	Gln	Val	Thr 200	Gly	Pro	Gln	Glu	Ser 205	Lys	Ile	Val
Gly	Ala 210	Leu	Glu	Val	Leu	Gly 215	Lys	Lys	Ala	Asp	Ile 220	Val	Ile	Ala	Asn
Gln 225	Asn	Gly	Ile	Thr	Leu 230	Asn	Gly	Val	Arg	Thr 235	Ile	Asn	Ser	Asp	Arg 240
Phe	Val	Ala	Thr	Thr 245	Ser	Glu	Leu	Ile	Asp 250	Pro	Asn	Gln	Met	Met 255	Leu
Lys	Val	Thr	Lys 260	Gly	Asn	Val	Ile	11e 265	Asp	Ile	Asp	Gly	Phe 270	Ser	Thr
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Gln	Ser 290	Ile	Thr	Ser	Gly	Asp 295	Asn	Ser	Glu	Ala	Lys 300	Thr	Asp	Val	Thr
Leu 305	Ile	Ala	Gly	Ser	Ser 310	Glu	Tyr	Asp	Leu	Ser 315	Ьуs	His	Glu	Leu	Lys 320
Lys	Thr	Ser	Gly	Glu 325	Asn	Val	Ser	Asn	Asp 330	Val	Ile	Ala	Ile	Thr 335	Gly
Ser	Ser	Thr	Gly 340	Ala	Met	His	Gly	Lys 345	Asn	Ile	Lys	Leu	Ile 350	Val	Thr
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Ala	Leu 450	Ser	Ile	Glu	Gln	Asn 455	Ala	Lys	Leu	Val	Ala 460	Lys	Lys	Ile	Asp
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acc Thr	gcg Ala 210	tta Leu	att Ile	gaa Glu	aaa Lys	gtg Val 215	aaa Lys	gcg Ala	att Ile	gca Ala	gaa Glu 220	gcg Ala	cgt Arg	tta Leu	ggc Gly	672

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aac Asn	ttc Phe	ccg Pro 355	cca Pro	tat Tyr	tct Ser	gtg Val	ggt Gly 360	gaa Glu	acc Thr	ggt Gly	atg Met	att Ile 365	ggt Gly	tca Ser	cca Pro	1104
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gtg Val	ctt Leu 450	tca Ser	gac Asp	atc Ile	tta Leu	ggt Gly 455	gat Asp	gaa Glu	gat Asp	cac His	tta Leu 460	ggt Gly	gac Asp	atg Met	gac Asp	1392
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360 365 Lys Arg Arg Glu Ile Gly His Gly Arg Leu Ala Lys Arg Gly Val Ala 375 Ala Val Met Pro Thr Leu Ala Glu Phe Pro Tyr Val Val Arg Val Val Ser Glu Ile Thr Glu Ser Asn Gly Ser Ser Ser Met Ala Ser Val Cys Gly Ala Ser Leu Ala Leu Met Asp Ala Gly Val Pro Ile Lys Ala Ala Val Ala Gly Ile Ala Met Gly Leu Val Lys Glu Asp Glu Lys Phe Val Val Leu Ser Asp Ile Leu Gly Asp Glu Asp His Leu Gly Asp Met Asp Phe Lys Val Ala Gly Thr Arg Thr Gly Val Thr Ala Leu Gln Met Asp Ile Lys Ile Glu Gly Ile Thr Ala Glu Ile Met Gln Ile Ala Leu Asn Gln Ala Lys Ser Ala Arg Leu His Ile Leu Gly Val Met Glu Gln Ala Ile Pro Ala Pro Arg Ala Asp Ile Ser Asp Phe Ala Pro Arg Ile Tyr Thr Met Lys Ile Asp Pro Lys Lys Ile Lys Asp Val Ile Gly Lys Gly Gly Ala Thr Ile Arg Ala Leu Thr Glu Glu Thr Gly Thr Ser Ile Asp Ile Asp Asp Asp Gly Thr Val Lys Ile Ala Ala Val Asp Gly Asn Ser Ala Lys Glu Val Met Ala Arg Ile Glu Asp Ile Thr Ala Glu Val Glu Ala Gly Ala Val Tyr Lys Gly Lys Val Thr Arg Leu Ala Asp Phe Gly Ala Phe Val Ser Ile Val Gly Asn Lys Glu Gly Leu Val His Ile Ser Gln Ile Ala Glu Glu Arg Val Glu Lys Val Ser Asp Tyr Leu Ala Val Gly Gln Glu Val Thr Val Lys Val Val Glu Ile Asp Arg Gln Gly Arg Ile Arg Leu Thr Met Lys Glu Val Ala Pro Lys Gln Glu His Val Asp 665 Ser Val Val Ala Asp Val Ala Ala Glu Glu Asn Ala

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Ile Tyr Asp Ala Leu Thr Leu Leu Gln His Arg Gly Gln Asp Ala Ala
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ggg att gta acc gta gat gat gaa aac cga ttc cgc ttg cgt aaa gcg
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Asn Gly Leu Val Ser Asp Val Phe Glu Gln Val His Met Leu Arg Leu
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caa ggc aat gct ggc att gga cat gtt cgt tat cct acg gct ggg agc
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Ser Ser Val Ser Glu Ala Gln Pro Phe Tyr Val Asn Ser Pro Tyr Gly
tta acc tta gtg cat aat ggt aac ttg acc aat tca agt gaa tta aaa
Leu Thr Leu Val His Asn Gly Asn Leu Thr Asn Ser Ser Glu Leu Lys
gaa aag tta ttt ogt oto goa ogt ogo oat gta aat acc aat toa gat
Glu Lys Leu Phe Arg Leu Ala Arg Arg His Val Asn Thr Asn Ser Asp
tot gaa tta tta ctc aat atc tta gcc aat cac ctt gat cac ttc gaa
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Ser Glu Leu Leu Asn Ile Leu Ala Asn His Leu Asp His Phe Glu
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Lys Tyr Gln Leu Asp Pro Gln Asp Val Phe Ser Ala Val Lys Gln Thr
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His Gln Asp Ile Arg Gly Ala Tyr Ala Cys Ile Ala Met Ile Ile Gly
cat ggt atg gtc gcg ttt cgt gat ccg aac ggt atc cgt ccg tta gtg
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His Gly Met Val Ala Phe Arg Asp Pro Asn Gly Ile Arg Pro Leu Val
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Lys 145	Tyr	Gln	Leu	Asp	Pro 150	Gln	Asp	Val	Phe	Ser 155	Ala	Va1	Lys	Gln	Thr 160	
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Glu Trp Ala Asp Val Asp Asp Ile Asp Val Val Ile Pro Val Pro Glu
Thr Ser Asn Asp Ile Ala Leu Arg Ile Ala Arg Val Leu Asn Lys Pro
Tyr Arg Gln Gly Phe Val Lys Asn Arg Tyr Val Gly Arg Thr Phe Ile
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Asp Lys Leu Ile Phe Gln Asp Leu Asp Ala Leu Thr Gly Ser Val Gln
Gln Glu Asn Pro Ser Ile Gln Asp Phe Asp Cys Ser Val Phe Thr Gly
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			aaa Lys												
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Ser Glu Gln Asp		Thr Tle Le		Ara Tur Cue	
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Phe Ala Ile Glu 195	Thr Ala	Met Arg Al 200	a Gly Glu Ile	Ala Ser Ile 205	Lys
Trp Asp Asn Val		Glu Lys Ar 215	g Ile Val His 220	Leu Pro Thr	Thr
Lys Asn Gly His 225	Ser Arg 230	Asp Val Pr	o Leu Ser Gln 235	Arg Ala Val	Ala 240
Leu Ile Leu Lys	Met Lys 245	Glu Val Gl	u Asn Gly Asp 250	Leu Val Phe 255	
Thr Thr Pro Glu 260		Ser Thr Th 26		Leu Lys Lys 270	Glu
Cys Gly Leu Glu 275	His Leu	His Phe Hi 280	s Asp Thr Arg	Arg Glu Ala 285	Leu
Thr Arg Leu Ser 290		Val Asp Va 295	l Met Thr Leu 300	Ala Lys Ile	Ser
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atg tct gaa gaa Met Ser Glu Glu 1 gca att cgc aca Ala Ile Arg Thr	Tyr Leu I 5 att caa a Ile Gln s	His Gly Vai agt cta tca Ser Leu Se: 29 gac aat gas	L Lys Val Thr 10 acc gca gtc Thr Ala Val	Glu Ile Asn 15 atc ggt att Ile Gly Ile 30 ctc aat gaa	gtc 96 Val

212

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Cys Thr Ala Asn Asp Ala Asp Asn Glu Thr Phe Pro Leu Asn Glu Pro $35 \hspace{1cm} 40 \hspace{1cm} 45$	
Val Leu Ile Thr Asn Val Ala Ala Ala Ile Gly Lys Ala Gly Lys Gln $_{\mbox{50}}$	
Gly Thr Leu Ser Arg Ala Leu Asp Gly Ile Ser Asp Val Val Asn Cys 65 70 75 80	
Lys Val Ile Val Val Arg Val Gln Glu Ser Ala Gln Glu Asp Glu Glu 85 90 95	
Thr Lys Ala Ser Glu Met Asn Thr Ala Ile Ile Gly Thr Ile Thr Glu 100 $$105$$	
Glu Gly Gln Tyr Thr Gly Leu Lys Ala Leu Leu Ile Ala Lys Asn Lys 115 120 125	
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Glu Val Tyr Thr Arg Thr Ala Gln Ile Leu Lys Asp Thr Ile Ala Gly
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Gly Tyr Leu Ile Gly Gly Lys Ala Trp Leu Asn Lys Glu Leu Asn Ser
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Leu Val Pro Val Ala Glu Thr Ile Asn Ser Ala Val Gly Asn Ala Ser
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Asn Thr Lys Asn Asn Gln Asn Glu Asn Tyr Ala Lys Ala His Ala Glu Gly Asn Phe Thr Val Lys Gly Gly Lys His Val Ile Ile Gly Lys Glu Val Lys Ala Asn Lys Ala Val Asp Ile Gln Ala Gln Glu Thr Thr Val Arg Gln Asn Ala Lys Leu Thr Ala Lys Thr Ser Ala Lys Ile Thr Ala Ser Lys Ser Val Asn Leu Glu Asp Asn Ala Lys Leu Ile Ala Asn Glu Leu Ser Thr Thr Thr Asn Lys Leu Thr Asn Lys Gly Ser Ile Tyr Gly Lys Lys Val Thr Leu Asp Ala Asp Asn Leu Val Asn Ser Lys Glu Ile Tyr Ala Ser Ser Glu Leu Asp Ile Gln Thr Lys Gly Arg Asp Leu Leu Leu Glu Asp Gly Val Asn Gln Pro Leu Ser Phe Leu Lys Gly Ala Ser Leu Leu Ala Pro Gly Phe Val Asn Thr Gly Leu Ile His Ser Asn Gly Asn Ala Lys Leu Thr Phe Lys Asp Asp Thr Ser Phe Val Thr Glu Gly Asn Asn Phe Ile Thr Ala Lys Asp Asn Leu Glu Ile Thr Ala Lys Asn Val Gln Ile Asp Gln Ala Lys Asn Ile Gln Leu Asn Ala Asn Ile Thr Ile Asn Thr Lys Ser Gly Phe Val Asn Tyr Gly Thr Leu Ala Ser Ala Gln Asn Leu Thr Ile Asn Thr Glu Gln Gly Ser Ile Tyr Asn Ile Gly Gly Ile Leu Gly Ala Gly Lys Ser Leu Asn Leu Ser Ala Lys Arg Gly Glu Asn Gln Gly Gly Tyr Leu Ile Asn Gln Gly Lys Ser Leu Leu His Ser Glu Gly Ala Met Asn Leu Thr Ala Asp Arg Thr Val Tyr Asn Leu 665 Gly Asn Ile Phe Ala Lys Gly Asp Ala Thr Ile Asn Ala Asn Ala Leu Ile Asn Asp Val Thr Leu Thr Gly Arg Leu Glu Tyr Gln Asp Leu Lys Lys Asp Tyr Thr Arg Tyr Tyr Arg Ile Asn Glu Thr Ala Lys His Gly

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221

Cys Asn His

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Ile Thr Cys Cys Val Asn His Asn Gly Phe Arg Phe Trp Gly Leu Arg
Thr Thr Ala Glu Asp Pro Leu Phe Lys Phe Glu Val Tyr Thr Arg Thr
Ala Gln Ile Leu Lys Asp Thr Ile Ala Gly Ala Phe Asp Trp Ala Val
Asp Lys Asp Ile Ser Val Thr Leu Val Lys Asp Ile Ile Glu Ala Ile
Asn Ala Lys Trp Arg Asp Tyr Thr Thr Lys Gly Tyr Leu Ile Gly Gly
Lys Ala Trp Leu Asn Lys Glu Leu Asn Ser Ala Thr Asn Leu Lys Asp
Ala Lys Leu Leu Ile Ser Tyr Asp Tyr His Pro Val Pro Pro Leu Glu
Gln Leu Gly Phe Asn Gln Tyr Ile Ser Asp Glu Tyr Leu Val Asp Phe
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Asp Lys Phe Lys Ile Leu Ser
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                     5
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gaa att gga t Glu Ile Gly L					
cat aaa gta g His Lys Val A 35					
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caa tot tto a Gln Ser Phe Th					
gat ggt ttt to Asp Gly Phe So	ca att aaa g er Ile Lys (85	gga aaa gac Gly Lys Asp	ttg tta cct Leu Leu Pro 90	gga tat caa Gly Tyr Gln 95	agt 288 Ser
att caa act co					
aat ttg gga g Asn Leu Gly G 115					
gga aaa agc g Gly Lys Ser G 130	ly Lys Arg C	ggt gcg agt Gly Ala Ser 135	agt aat gtc Ser Asn Val 140	agc tta ctt Ser Leu Leu	aaa 432 Lys
tcg ttt aat at Ser Phe Asn Me 145					
agt tat tat to Ser Tyr Tyr Se					
tcc gaa tta g Ser Glu Leu G					
tat gca aca ca Tyr Ala Thr H: 195					
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 His Lys Val Ala Thr Asn Pro Phe Leu Ala Leu Asp Leu Ser Leu Gly
 Asn Phe Tyr Met Arg Gly Thr Ala Gly Ile Ser Glu Ile Gly Tyr Glu
Gln Ser Phe Thr Asp Asn Phe Ser Val Ser Leu Phe Val Asn Pro Phe
Asp Gly Phe Ser Ile Lys Gly Lys Asp Leu Leu Pro Gly Tyr Gln Ser
Ile Gln Thr Arg Lys Thr Gln Phe Ala Phe Gly Trp Gly Leu Asn Tyr
Asn Leu Gly Gly Leu Phe Gly Leu Asn Asp Thr Phe Ile Ser Leu Glu
Gly Lys Ser Gly Lys Arg Gly Ala Ser Ser Asn Val Ser Leu Leu Lys
Ser Phe Asn Met Thr Lys Asn Trp Lys Val Ser Pro Tyr Ile Gly Ser
Ser Tyr Tyr Ser Ser Lys Tyr Thr Asp Tyr Tyr Phe Gly Ile Lys Gln
Ser Glu Leu Gly Asn Lys Ile Thr Ser Val Tyr Lys Pro Lys Ala Ala
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atg gct cgc cag att tta tca gcg gcg gag ttg ctc att gca aag gaa
Met Ala Arg Gln Ile Leu Ser Ala Ala Glu Leu Leu Ile Ala Lys Glu
 ggt ttg caa aat tta tcg atg agg aaa atc gca agt gaa gcc ggt atc
Gly Leu Gln Asn Leu Ser Met Arg Lys Ile Ala Ser Glu Ala Gly Ile
 gca aca ggc acg ctt tat ctc tat ttc aaa acg aaa gac gag tta ctg
                                                                       192
 Ala Thr Gly Thr Leu Tyr Leu Tyr Phe Lys Thr Lys Asp Glu Leu Leu
gat tgt ttg gcg gaa caa tta cat gaa cga tat tat cgt tat ctg aat
 Asp Cys Leu Ala Glu Gln Leu His Glu Arg Tyr Tyr Arg Tyr Leu Asn
at
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Met Ala Arg Gln Ile Leu Ser Ala Ala Glu Leu Leu Ile Ala Lys Glu
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Ala Thr Gly Thr Leu Tyr Leu Tyr Phe Lys Thr Lys Asp Glu Leu Leu
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caa gta gat att ggt gca caa gta tct ggg cag att aag cat att tta
Gln Val Asp Ile Gly Ala Gln Val Ser Gly Gln Ile Lys His Ile Leu
gta caa gaa gga cag aag gtt aaa aaa ggt gaq cta tta qct qta att
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	aat Asn															240
	caa Gln															288
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aaa Lys	gca Ala	gaa Glu 115	ctt Leu	caa Gln	att Ile	gcg Ala	caa Gln 120	aat Asn	aat Asn	cta Leu	gat Asp	atc Ile 125	gct Ala	aaa Lys	atc Ile	384
aga Arg	gtg Val 130	gaa Glu	aaa Lys	gct Ala	gaa Glu	acc Thr 135	gaa Glu	cta Leu	gga Gly	tat Tyr	aca Thr 140	gaa Glu	att Ile	cgt Arg	tct Ser	432
cca Pro 145	ctt Leu	gat Asp	gca Ala	aca Thr	gta Val 150	att Ile	tca Ser	gta Val	ttt Phe	gcg Ala 155	caa Gln	aat Asn	ggt Gly	caa Gln	act Thr 160	480
tta Leu	gtc Val	acc Thr	acc	caa Gln 165	caa Gln	gta Val	cca Pro	gtg Val	ctg Leu 170	atg Met	aaa Lys	tta Leu	gct Ala	aat Asn 175	at	527
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	Val		20					25	-				30			
Val	Gln	Glu 35	Gly	Gln	Lys	Val	Lys 40	Lys	Gly	Glu	Leu	Leu 45	Ala	Val	Ile	
Asp	Pro 50	Arg	Leu	Ala	Glu	Thr 55	Glu	Leu	Lys	Leu	Ala 60	Lys	Ala	Glu	Leu	
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Lys Ala Glu Leu Gln Ile Ala Gln Asn Asn Leu Asp Ile Ala Lys Ile
Arg Val Glu Lys Ala Glu Thr Glu Leu Gly Tyr Thr Glu Ile Arg Ser
Pro Leu Asp Ala Thr Val Ile Ser Val Phe Ala Gln Asn Gly Gln Thr
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act caa aaa atc acc aaa gca atg gaa atg gtt gct acc tct aaa atg
Thr Gln Lys Ile Thr Lys Ala Met Glu Met Val Ala Thr Ser Lys Met
cgt aaa acg caa gag cgt atg gct gcc agt cgt cct tat tcg gaa aca
Arg Lys Thr Gln Glu Arg Met Ala Ala Ser Arg Pro Tyr Ser Glu Thr
atc cgt aag gtg att agc cat att gcg aaa gga agc att ggt tat aag
                                                                         192
Ile Arg Lys Val Ile Ser His Ile Ala Lys Gly Ser Ile Gly Tyr Lys
cac ccg ttt tta act gaa cgt gat att aaa aaa gta ggc tat ctt gtc
                                                                         240
His Pro Phe Leu Thr Glu Arg Asp Ile Lys Lys Val Gly Tyr Leu Val
gtt tcg acc gat cgc ggt tta tgc ggt ggc ctt aat atc aat tta ttc
Val Ser Thr Asp Arg Gly Leu Cys Gly Gly Leu Asn Ile Asn Leu Phe
aaa gcg act ttg aat gaa ttt aaa acg tgg aaa gat aaa gac gtt agt
Lys Ala Thr Leu Asn Glu Phe Lys Thr Trp Lys Asp Lys Asp Val Ser
                                                                        336
             100
gtt gag ctt ggt tta gta ggg tcg aaa ggc gta agc ttt tac caa aat
Val Glu Leu Gly Leu Val Gly Ser Lys Gly Val Ser Phe Tyr Gln Asn
cta ggc tta aac gtg aga tct caa gta acg gga tta ggc gat aat ccg
                                                                        432
Leu Gly Leu Asn Val Arg Ser Gln Val Thr Gly Leu Gly Asp Asn Pro
gaa atg gaa cgt atc gtg ggc gca gtt aat gaa atg att aat gcg ttc
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Glu 145	Met	Glu	Arg	Ile	Val 150	Gly	Ala	Val	Asn	Glu 155	Met	Ile	Asn	Ala	Phe 160	
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aat Asn	acg Thr	atg Met	tca Ser 180	caa Gln	aaa Lys	cct Pro	gtt Val	atc Ile 185	gca Ala	cag Gln	tta Leu	ctt Leu	ccg Pro 190	tta Leu	cct Pro	576
aaa Lys	cta Leu	gat Asp 195	gac Asp	gat Asp	gaa Glu	tta Leu	gat Asp 200	acg Thr	aaa Lys	ggt Gly	tca Ser	tgg Trp 205	gat Asp	tat Tyr	att Ile	624
tat Tyr	gaa Glu 210	ccg Pro	aat Asn	cca Pro	caa Gln	gtt Val 215	tta Leu	ttg Leu	gat Asp	agt Ser	tta Leu 220	ctt Leu	gtt Val	cgt Arg	tat Tyr	672
tta Leu 225	gaa Glu	act Thr	cag Gln	gta Val	tac Tyr 230	caa Gln	gca Ala	gtt Val	gta Val	gat Asp 235	aac Asn	cta Leu	gct Ala	tct Ser	gaa Glu 240	720
caa Gln	gcc Ala	gct Ala	cga Arg	atg Met 245	gta Val	gcg Ala	atg Met	aaa Lys	gcc Ala 250	gca Ala	aca Thr	gat Asp	aat Asn	gcg Ala 255	ggt Gly	768
aca Thr	tta Leu	atc Ile	gat Asp 260	gaa Glu	tta Leu	caa Gln	tta Leu	gtg Val 265	tat Tyr	aac Asn	aaa Lys	gct Ala	cgc Arg 270	caa Gln	gca Ala	816
agc Ser	att Ile	aca Thr 275	aat Asn	gaa Glu	tta Leu	aac Asn	gaa Glu 280	att Ile	gtt Val	gcg Ala	ggt Gly	gcc Ala 285	gca Ala	gca Ala	att Ile	864
taa																867
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Thr	Gln	Lys	Ile 20	Thr	Lys	Ala	Met	Glu 25	Met	Val	Ala	Thr	Ser 30	Lys	Met	
Arg	Lys	Thr 35	Gln	Glu	Arg	Met	Ala 40	Ala	Ser	Arg	Pro	Tyr 45	Ser	Glu	Thr	
Ile	Arg 50	Lys	Val	Ile	Ser	His 55	Ile	Ala	Lys	Gly	Ser 60	Ile	Gly	Tyr	Lys	
His 65	Pro	Phe	Leu	Thr	Glu 70	Arg	Asp	Ile	Lys	Lys 75	Val	Gly	Tyr	Leu	Val 80	
Val	Ser	Thr	Asp	Arg 85	Gly	Leu	Cys	Gly	Gly 90	Leu	Asn	Ile	Asn	Leu 95	Phe	

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Glu Met Glu Arg Ile Val Gly Ala Val Asn Glu Met Ile Asn Ala Phe
Arg Asn Gly Glu Val Asp Ala Val Tyr Val Ala Tyr Asn Arg Phe Glu
Asn Thr Met Ser Gln Lys Pro Val Ile Ala Gln Leu Leu Pro Leu Pro
Lys Leu Asp Asp Asp Glu Leu Asp Thr Lys Gly Ser Trp Asp Tyr Ile
Tyr Glu Pro Asn Pro Gln Val Leu Leu Asp Ser Leu Leu Val Arg Tyr
Leu Glu Thr Gln Val Tyr Gln Ala Val Val Asp Asn Leu Ala Ser Glu
225
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gat ttt gct tta gaa caa ggt cag ttg gac aaa tgg caa gaa atg tta
Asp Phe Ala Leu Glu Gln Gly Gln Leu Asp Lys Trp Gln Glu Met Leu
cag ttt tcg gca ttc gtt gct gaa aac gaa caa gtg gcg gaa tat att
                                                                   144
Gln Phe Ser Ala Phe Val Ala Glu Asn Glu Gln Val Ala Glu Tyr Ile
                             40
aat tot too ott goa ago ggt cag att tot gaa act ttt atc aaa atc
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231

As n Ser Ser Leu Ala Ser Gly Gln Ile Ser Glu Thr Phe Ile Lys Ile tog gog gac caa ctt gat caa tat gog caa aat ttt att cgt gta atg Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 65 gct gaa aat aaa cgt ctg gct gtg ttg cct atg gtt ttt gat act ttc 76 gct gaa aat aaa cgt ctg gct gtg ttg cct atg gtt ttt gat act ttc Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 85 gta tca tta cga gcg gaa cat gaa gcg gta aaa gat gta aca att gtt Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val 100 tcg gca aac gaa tta agt caa gca caa gaa gat aaa atc gca aaa gcg Ser Ala Asn Glu Leu Ser Gln Ala Gln Glu Asp Lys Ile Ala Lys Ala det Glu Lys Arg Leu Gly Gln Lys Val Arg Leu Thr Asn Gln Ile Asp 130 aca agc ctg att gca gcg gct att att aaa tac gat gat gtt att Asn Ser Leu Ile Ala Gly Val Ile Ile Lys Tyr Asp Asp Val Val Ile 145 gat ggt agt agc cgc ggt cag tta aat cgc tta gcg tca gcg tta gc Asp Gly Ser Ser Arg Gly Gln Leu Asn Arg Leu Ala Ser Ala Leu c210> 135 c310> 135 c310> 135 c311> 177 c312> PRT c311> 178 c311> 17																	
Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 65 65 65 65 66 65 66 65 66 66 67 67	Asn		Ser	Leu	Ala	Ser		Gln	Ile	Ser	Glu		Phe	Ile	Lys	Ile	
Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 850	Cys					Asp					Asn					Met	240
Val Ser Leu Arg Ala Glu His Glu Ala Lys Asp Val Thr Ile Val 1005 1005 1005 1005 1006 1007 1006 1007 1007 1008					Arg					Pro					Thr		288
Ser Ala Asn Glu Leu Ser Gln Ala Gln Glu Asp Lys Ile Ala Lys Ala 115 126 127 128 129 130 130 131 130 131 130 131 130 131 130 131 130 131 130 131 130 131 130 130 131 130 1				Arg					Ala					Thr			336
Met Glu Lys Arg Leu Gly Gln Lys Val Arg Leu Thr Asn Gln Ile Asp 130 aac agc ctg att gca ggc gta att att aaa tac gat gat gtt gtt att Asn Ser Leu Ile Ala Gly Val Ile Ile Lys Tyr Asp Asp Val Val Ile 150 gat ggt agt agc cgc ggt cag tta aat cgc tta gcg tca gcg ttg agc Asp Gly Ser Ser Arg Gly Gln Leu Asn Arg Leu Ala Ser Ala Leu Ser 165 ttg taa Leu <pre></pre>			Asn					Ala					Ile				384
Asn Ser Leu Ile Ala Giy Val Ile Ile Lys Tyr Asp Asp Val Val Ile 150 160 gat ggt agt agc cgc ggt cag tta aat cgc tta gcg tca gcg ttg agc Asp Gly Ser Ser Arg Gly Gln Leu Asn Arg Leu Ala Ser Ala Leu Ser 165 170 ttg taa 4210> 135 4211> 177 4212> PKT 4213> Actinobacillus pleuropneumoniae 4400> 135 Met Ser Glu Leu Ser Thr Val Ala Arg Pro Tyr Ala Lys Ala Ala Phe 1 Asp Phe Ala Leu Glu Gln Gly Gln Leu Asp Lys Trp Gln Glu Met Leu 20 Gln Phe Ser Ala Phe Val Ala Glu Asn Glu Gln Val Ala Glu Tyr Ile 35 Asn Ser Ser Leu Ala Ser Gly Gln Ile Ser Glu Thr Phe Ile Lys Ile 50 Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 70 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val	atg Met	Glu	aaa Lys	cgc Arg	tta Leu	ggt Gly	Gln	aaa Lys	gtt Val	cgt Arg	tta Leu	Thr	aac Asn	caa Gln	atc Ile	gat Asp	432
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Met Ser Glu Leu Ser Thr Val Ala Arg Pro Tyr Ala Lys Ala Ala Phe 1 10 15 Asp Phe Ala Leu Glu Gln Gly Gln Leu Asp Lys Trp Gln Glu Met Leu 20 30 Gln Phe Ser Ala Phe Val Ala Glu Asn Glu Gln Val Ala Glu Tyr Ile 35 45 Asn Ser Ser Leu Ala Ser Gly Gln Ile Ser Glu Thr Phe 11e Lys Ile 50 Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 70 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val	<21 <21	l> 17 2> PF	77 ?T	baci	llus	ple	europ	neum	nonia	ne .							
20 25 30 Gln Phe Ser Ala Phe Val Ala Glu Asn Glu Gln Val Ala Glu Tyr Ile 35 40 Asn Ser Ser Leu Ala Ser Gly Gln Ile Ser Glu Thr Phe Ile Lys Ile 50 60 Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 70 80 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 95 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val				Leu		Thr	Val	Ala	Arg		Tyr	Ala	Lys	Ala		Phe	
Asn Ser Ser Leu Ala Ser Gly Gln Ile Ser Glu Thr Phe Ile Lys Ile 50 Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 70 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val	Asp	Phe	Ala		Glu	Gln	Gly	Gln		Asp	Lys	Trp	Gln		Met	Leu	
50 55 60 Cys Gly Asp Gln Leu Asp Gln Tyr Gly Gln Asn Phe Ile Arg Val Met 65 70 80 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 95 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val	Gln	Phe		Ala	Phe	Val	Ala		Asn	Glu	Gln	Val		Glu	Tyr	Ile	
65 70 75 80 Ala Glu Asn Lys Arg Leu Ala Val Leu Pro Met Val Phe Asp Thr Phe 85 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val	Asn		Ser	Leu	Ala	Ser		Gln	Ile	Ser	Glu		Phe	Ile	Lys	Ile	
85 90 95 Val Ser Leu Arg Ala Glu His Glu Ala Val Lys Asp Val Thr Ile Val		Gly	Asp	Gln	Leu		Gln	Tyr	Gly	Gln		Phe	Ile	Arg	Val		
	Ala	Glu	Asn	Lys		Leu	Ala	Val	Leu		Met	Val	Phe	Asp		Phe	
	Val	Ser	Leu		Ala	Glu	His	Glu		Val	Lys	Asp	Val		Ile	Val	

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Ser Ala Asn Glu Leu Ser Gln Ala Gln Glu Asp Lys Ile Ala Lys Ala
          115
Met Glu Lys Arg Leu Gly Gln Lys Val Arg Leu Thr Asn Gln Ile Asp
Asn Ser Leu Ile Ala Gly Val Ile Ile Lys Tyr Asp Asp Val Val Ile
145
Asp Gly Ser Ser Arg Gly Gln Leu Asn Arg Leu Ala Ser Ala Leu Ser
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Leu
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atg cag gaa gaa gtc gct aat ttc gcc gat cct gcg gac cgc gcc act
Met Gln Glu Glu Val Ala Asn Phe Ala Asp Pro Ala Asp Arg Ala Thr
cag gaa gaa gaa ttc agt ctt gaa tta aga aac cgt gac cgt gag cgt
                                                                          144
Gln Glu Glu Glu Phe Ser Leu Glu Leu Arg Asn Arg Asp Arg Glu Arg
aaa ttg ctt aag aag att gag caa acg tta aat agc att gcc gaa gac
                                                                          192
Lys Leu Leu Lys Lys Ile Glu Gln Thr Leu Asn Ser Ile Ala Glu Asp
gaa tac ggc tat tgc gaa act tgc ggt gtt gaa atc ggt tta cgt cgt
                                                                          240
Glu Tyr Gly Tyr Cys Glu Thr Cys Gly Val Glu Ile Gly Leu Arg Arg
                                             75
tta gaa gcg cgc ccg acc gcg gat atg tgt atc gat tgc aaa aca ctt
                                                                          288
Leu Glu Ala Arg Pro Thr Ala Asp Met Cys Ile Asp Cys Lys Thr Leu
gcg gaa atc cgt gaa aag caa atg ggc tta taa
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Ala Glu Ile Arg Glu Lys Gln Met Gly Leu
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<211> 106
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<213> Actinobacillus pleuropneumoniae
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Lys Leu Leu Lys Lys Ile Glu Gln Thr Leu Asn Ser Ile Ala Glu Asp
Glu Tyr Gly Tyr Cys Glu Thr Cys Gly Val Glu Ile Gly Leu Arg Arg
 65
Leu Glu Ala Arg Pro Thr Ala Asp Met Cys Ile Asp Cys Lys Thr Leu
Ala Glu Ile Arg Glu Lys Gln Met Gly Leu
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tta g Leu (ggc 31y	tta Leu	tta Leu 20	cta Leu	tta Leu	atg Met	agt Ser	gtt Val 25	gtg Val	ttg Leu	gta Val	tgg Trp	aaa Lys 30	att Ile	att Ile	96
gaa d Glu A	ege Arg	gta Val 35	ctt Leu	ttc Phe	tac Tyr	aaa Lys	caa Gln 40	ttg Leu	gat Asp	gtg Val	acc Thr	aaa Lys 45	tat Tyr	gac Asp	acg Thr	144
cta c Leu C																192
act a Thr I 65	atc Ile	ggt Gly	gcc Ala	aac Asn	gcc Ala 70	cct Pro	tat Tyr	atc Ile	ggt Gly	tta Leu 75	tta Leu	gga Gly	acc Thr	gta Val	tta Leu 80	240
ggg a	atc [le	tta Leu	ctt Leu	acc Thr 85	ttc Phe	tat Tyr	cat His	tta Leu	999 Gly 90	cat His	tcc ser	ggc Gly	ggt Gly	gat Asp 95	att Ile	288
gac c Asp A	gcc	gca Ala	tcc Ser 100	att Ile	atg Met	gtt Val	cac His	ctt Leu 105	tcg Ser	ctt Leu	gca Ala	tta Leu	aaa Lys 110	gca Ala	acc Thr	336
gca g Ala A	gcc Ala	ggt Gly 115	atc Ile	tta Leu	gtc Val	gct Ala	att Ile 120	ccg Pro	gca Ala	atg Met	atg Met	ttc Phe 125	tac Tyr	agc Ser	ggt Gly	384
ttt a Phe A	aac Asn 130	cgt Arg	aaa Lys	gtg Val	gat Asp	gaa Glu 135	agc Ser	aaa Lys	ctt Leu	aaa Lys	tgg Trp 140	caa Gln	gcg Ala	att Ile	caa Gln	432
gct c Ala A 145						taa										453
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Leu G	ЗІУ	Leu	Leu 20	Leu	Leu	Met	Ser	Val 25	Val	Leu	Val	Trp	Lys 30	Ile	Ile	
Glu A	arg	Val 35	Leu	Phe	Tyr	Lys	Gln 40	Leu	Asp	Val	Thr	Lys 45	Tyr	Asp	Thr	
Leu G	1n 50	Asp	Leu	Glu	Ile	Asp 55	Thr	Thr	Arg	Asn	Leu 60	Thr	Thr	Ile	Ser	
Thr I 65	1e	Gly	Ala	Asn	Ala 70	Pro	Tyr	Ile	Gly	Leu 75	Leu	Gly	Thr	Val	Leu 80	

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Asp Ala Ala Ser Ile Met Val His Leu Ser Leu Ala Leu Lys Ala Thr
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gtt tca gct caa act gca ttt gca gcg gat caa aaa ttc att gac gat
Val Ser Ala Gln Thr Ala Phe Ala Ala Asp Gln Lys Phe Ile Asp Asp
tca tca tat gca gtc ggc gta ttg atg ggt aaa aat atc gaa ggc gtc
                                                                   144
Ser Ser Tyr Ala Val Gly Val Leu Met Gly Lys Asn Ile Glu Gly Val
gtt gaa toa caa aaa gaa att ttt tot tat aac caa gat aaa atc ttg
                                                                   192
Val Glu Ser Gln Lys Glu Ile Phe Ser Tyr Asn Gln Asp Lys Ile Leu
gcg ggt gtc caa gat acc atc aaa aaa acc ggt aaa tta acc gat gaa
Ala Gly Val Gln Asp Thr Ile Lys Lys Thr Gly Lys Leu Thr Asp Glu
                     70
gat cta caa aaa caa tta aaa tcg ctt gat act tat ctt gca agt caa
Asp Leu Gln Lys Gln Leu Lys Ser Leu Asp Thr Tyr Leu Ala Ser Gln
                 85
                                     90
gaa agc aaa att gcg gcg gag aaa agc aaa gca acc gta gaa gcc ggt
                                                                  336
Glu Ser Lys Ile Ala Ala Glu Lys Ser Lys Ala Thr Val Glu Ala Gly
aat aaa ttt cgt acc gac tac gaa aaa caa agc ggc gtg aaa aaa acc
                                                                  384
Asn Lys Phe Arg Thr Asp Tyr Glu Lys Gln Ser Gly Val Lys Lys Thr
get tee ggt tta ett tat aaa att gaa aaa gee gge acg gge gaa teg
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Ala	Ser 130	Gly	Leu	Leu	Tyr	Lys 135		Glu	Lys	Ala	Gly 140		Gly	Glu	Ser	
cct Pro 145	aaa Lys	gcg Ala	gaa Glu	gat Asp	acc Thr 150	Val	aaa Lys	gtt Val	cac His	tat Tyr 155	aaa Lys	ggg Gly	aca Thr	tta Leu	acc Thr 160	480
gat Asp	ggt Gly	acg Thr	gta Val	ttc Phe 165	gat Asp	agc Ser	tca Ser	tac Tyr	gat Asp 170	cgc Arg	ggt Gly	gag Glu	ccg Pro	att Ile 175	gaa Glu	528
ttc Phe	caa Gln	tta Leu	aac Asn 180	caa Gln	tta Leu	att Ile	ccg Pro	ggt Gly 185	tgg Trp	att Ile	gaa Glu	gcg Ala	att Ile 190	cca Pro	atg Met	576
ttg Leu	aaa Lys	aaa Lys 195	ggc Gly	gga Gly	aaa Lys	atg Met	gaa Glu 200	atc Ile	gtc Val	gtt Val	ccg Pro	cct Pro 205	gaa Glu	ctt Leu	ggt Gly	624
					gca Ala											672
ttc Phe 225	gag Glu	att Ile	gaa Glu	ttg Leu	tta Leu 230	gat Asp	ttc Phe	aaa Lys	gcg Ala	gcc Ala 235	gaa Glu	gcg Ala	aaa Lys	aaa Lys	taa	720
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Met 1	Leu	Lys		5	Leu				10				_	15		
Met 1	Leu	Lys		5	Leu Ala				10				_	15		
Met 1 Val	Leu Ser	Lys Ala	Gln 20	5 Thr		Phe	Ala	Ala 25	10 Asp	Gln	Lys	Phe	Ile 30	15 Asp	Asp	
Met 1 Val Ser	Leu Ser Ser	Lys Ala Tyr 35	Gln 20 Ala	5 Thr Val	Ala	Phe Val	Ala Leu 40	Ala 25 Met	10 Asp Gly	Gln Lys	Lys Asn	Phe Ile 45	Ile 30 Glu	15 Asp Gly	Asp Val	
Met 1 Val Ser	Ser Ser Glu 50	Lys Ala Tyr 35 Ser	Gln 20 Ala Gln	5 Thr Val Lys	Ala Gly	Phe Val Ile 55	Ala Leu 40 Phe	Ala 25 Met Ser	10 Asp Gly Tyr	Gln Lys Asn	Lys Asn Gln 60	Phe Ile 45 Asp	Ile 30 Glu Lys	15 Asp Gly Ile	Asp Val Leu	
Met 1 Val Ser Val Ala 65	Ser Ser Glu 50	Lys Ala Tyr 35 Ser Val	Gln 20 Ala Gln Gln	5 Thr Val Lys Asp	Ala Gly Glu Thr	Phe Val Ile 55	Ala Leu 40 Phe Lys	Ala 25 Met Ser	Asp Gly Tyr	Gln Lys Asn Gly 75	Lys Asn Gln 60 Lys	Phe Ile 45 Asp	Ile 30 Glu Lys Thr	Asp Gly Ile Asp	Asp Val Leu Glu 80	
Met 1 Val Ser Val Ala 65 Asp	Ser Ser Glu 50 Gly Leu	Lys Ala Tyr 35 Ser Val	Gln 20 Ala Gln Gln Lys	5 Thr Val Lys Asp Gln 85	Ala Gly Glu Thr 70	Phe Val Ile 55 Ile Lys	Ala Leu 40 Phe Lys Ser	Ala 25 Met Ser Lys Leu	Asp Gly Tyr Thr Asp	Gln Lys Asn Gly 75 Thr	Lys Asn Gln 60 Lys	Phe Ile 45 Asp Leu Leu	Ile 30 Glu Lys Thr	Asp Gly Ile Asp Ser	Asp Val Leu Glu 80 Gln	
Met 1 Val Ser Val Ala 65 Asp Glu	Ser Ser Glu 50 Gly Leu Ser	Lys Ala Tyr 35 Ser Val Gln Lys	Gln 20 Ala Gln Gln Lys Ile 100	5 Thr Val Lys Asp Gln 85 Ala	Ala Gly Glu Thr 70 Leu	Phe Val Ile 55 Ile Lys Glu	Ala Leu 40 Phe Lys Ser	Ala 25 Met Ser Lys Leu Ser 105	10 Asp Gly Tyr Thr Asp 90 Lys	Gln Lys Asn Gly 75 Thr	Lys Asn Gln 60 Lys Tyr	Phe Ile 45 Asp Leu Leu Val	Ile 30 Glu Lys Thr Ala Glu 110	Asp Gly Ile Asp Ser 95 Ala	Asp Val Leu Glu 80 Gln Gly	
Met 1 Val Ser Val Ala 65 Asp Glu Asn	Ser Ser Glu 50 Gly Leu Ser	Lys Ala Tyr 35 Ser Val Gln Lys Phe	Gln 20 Ala Gln Gln Lys Ile 100 Arg	5 Thr Val Lys Asp Gln 85 Ala	Ala Gly Glu Thr 70 Leu Ala	Phe Val Ile 55 Ile Lys Glu Tyr	Ala Leu 40 Phe Lys Ser Lys Glu 120	Ala 25 Met Ser Lys Leu Ser 105	10 Asp Gly Tyr Thr Asp 90 Lys	Gln Lys Asn Gly 75 Thr Ala	Lys Asn Gln 60 Lys Tyr Thr	Phe Ile 45 Asp Leu Leu Val Val	Ile 30 Glu Lys Thr Ala Glu 110 Lys	15 Asp Gly Ile Asp Ser 95 Ala	Asp Val Leu Glu 80 Gln Gly Thr	

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Asp Gly Thr Val Phe Asp Ser Ser Tyr Asp Arg Gly Glu Pro Ile Glu
                                       170
Phe Gln Leu Asn Gln Leu Ile Pro Gly Trp Ile Glu Ala Ile Pro Met
Leu Lys Lys Gly Gly Lys Met Glu Ile Val Val Pro Pro Glu Leu Gly
Tyr Gly Glu Arg Gln Ala Gly Lys Ile Pro Ala Ser Ser Thr Leu Lys
Phe Glu Ile Glu Leu Leu Asp Phe Lys Ala Ala Glu Ala Lys Lys
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cct gaa gag att agt gtg ggg atg att att gcg gcg gtg aat gaa aat
Pro Glu Glu Ile Ser Val Gly Met Ile Ile Ala Ala Val Asn Glu Asn
                                                                      95
ctc gac gta acc aaa tgt aaa ggt agc ggc aac tgt agc aaa aac tct
                                                                      143
Leu Asp Val Thr Lys Cys Lys Gly Ser Gly Asn Cys Ser Lys Asn Ser
cag tgc tta acc cat cat tta tgg gaa cgt tta gaa gaa caa atc ggt
                                                                      191
Gln Cys Leu Thr His His Leu Trp Glu Arg Leu Glu Glu Gln Ile Gly
gtg ttt tta aat acg att act tta gcg gaa ctt gtt gaa gaa cat tcg
Val Phe Leu Asn Thr Ile Thr Leu Ala Glu Leu Val Glu Glu His Ser
                          70
gat cac gat tgt gaa aaa gaa cat tgc cac gat cat tca cac aaa cat
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Asp His Asp Cys Glu Lys Glu His Cys His Asp His Ser His Lys His
80
                      85
taa
                                                                      290
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 Asp Val Thr Lys Cys Lys Gly Ser Gly Asn Cys Ser Lys Asn Ser Gln
 Cys Leu Thr His His Leu Trp Glu Arg Leu Glu Glu Gln Ile Gly Val
 Phe Leu Asn Thr Ile Thr Leu Ala Glu Leu Val Glu Glu His Ser Asp
His Asp Cys Glu Lys Glu His Cys His Asp His Ser His Lys His
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age aag aaa gac geg aaa geg gea tta gaa geg act tta aat geg ate
Ser Lys Lys Asp Ala Lys Ala Ala Leu Glu Ala Thr Leu Asn Ala Ile
tct gaa agc cta aaa aat ggc gac acc gtt cag tta atc ggc ttc ggt
Ser Glu Ser Leu Lys Asn Gly Asp Thr Val Gln Leu Ile Gly Phe Gly
                                                                           144
act ttt aaa gta aac gag cgt aat gca cgt acg ggt cgt aac ccg cgt
                                                                           192
Thr Phe Lys Val Asn Glu Arg Asn Ala Arg Thr Gly Arg Asn Pro Arg
acc ggc gaa gaa atc aaa atc gca gca tct aaa gtg ccg gcg ttt gtt Thr Gly Glu Glu Ile Lys Ile Ala Ala Ser Lys Val Pro Ala Phe Val
                        70
gca ggt aaa gca tta aaa gat tta gta aaa taa
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Ala Gly Lys Ala Leu Lys Asp Leu Val Lys
                   85
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<213> Actinobacillus pleuropneumoniae

<400> 147

Met Asn Lys Thr Glu Leu Ile Asp Ala Ile Ala Ala Gly Ala Glu Leu

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Ser Glu Ser Leu Lys Asn Gly Asp Thr Val Gln Leu Ile Gly Phe Gly
Thr Phe Lys Val Asn Glu Arg Asn Ala Arg Thr Gly Arg Asn Pro Arg
Thr Gly Glu Glu Ile Lys Ile Ala Ala Ser Lys Val Pro Ala Phe Val
Ala Gly Lys Ala Leu Lys Asp Leu Val Lys
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gcc ggt tat tca gcg gca ttc cgt tgt gcc gac tta ggc tta gaa aca
Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu Thr
gta att gtc gaa cgt tat tca act ttg ggc ggt gta tgc tta aac gta
                                                                             144
Val Ile Val Glu Arg Tyr Ser Thr Leu Gly Gly Val Cys Leu Asn Val
ggt tgt att ccg tct aaa gca tta tta cac gtt gca aaa gtt atc gaa
                                                                             192
Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys Val Ile Glu
gaa gca aaa cac gca gag aaa aac ggt att act ttc ggt gag ccc aac Glu Ala Lys His Ala Glu Lys Asn Gly Ile Thr Phe Gly Glu Pro Asn
                                                                             240
att gat tta gat aaa gtg cgt gcg ggt aaa gaa gcg gtt gtt tct aaa
                                                                             288
Ile Asp Leu Asp Lys Val Arg Ala Gly Lys Glu Ala Val Val Ser Lys
tta acc ggc ggt tta gcg ggt atg gct aaa gca cgt aaa gta aca gta
                                                                             336
Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Ala Arg Lys Val Thr Val
                                     105
gtg gaa ggt tta gcg gcg ttt acc gat ccg aat act tta gta gct cgt
Val Glu Gly Leu Ala Ala Phe Thr Asp Pro Asn Thr Leu Val Ala Arg
                                                                             384
                                120
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gac cgt gac ggt aat ccg aca acg att aaa ttt gat tat gca att att
Asp Arg Asp Gly Asn Pro Thr Thr Ile Lys Phe Asp Tyr Ala Ile Ile
                         135
gca gcc ggt tot cgt ccg att cag ott ccg tto att cca cac gaa gat
                                                                       480
Ala Ala Gly Ser Arg Pro Ile Gln Leu Pro Phe Ile Pro His Glu Asp
ccg cgt gtg tgg gat tct acg gat gca ctt aaa tta aaa gaa gta ccc
Pro Arg Val Trp Asp Ser Thr Asp Ala Leu Lys Leu Lys Glu Val Pro
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gaa aaa att act cat tat ggg cc
                                                                      551
Glu Lys Ile Thr His Tyr Gly
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Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu Thr
Val·Ile Val Glu Arg Tyr Ser Thr Leu Gly Gly Val Cys Leu Asn Val
Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys Val Ile Glu
Glu Ala Lys His Ala Glu Lys Asn Gly Ile Thr Phe Gly Glu Pro Asn
Ile Asp Leu Asp Lys Val Arg Ala Gly Lys Glu Ala Val Val Ser Lys
Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Ala Arg Lys Val Thr Val
Val Glu Gly Leu Ala Ala Phe Thr Asp Pro Asn Thr Leu Val Ala Arg
Asp Arg Asp Gly Asn Pro Thr Thr Ile Lys Phe Asp Tyr Ala Ile Ile
Ala Ala Gly Ser Arg Pro Ile Gln Leu Pro Phe Ile Pro His Glu Asp
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241

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Ala Gln Ala Ala Pro Gln Gln Asn Thr Phe Tyr Ala Gly Ala Lys Ala
ggt tgg gcg tca ttc cat gat ggt atc gaa caa tta gat tca gct aaa
Gly Trp Ala Ser Phe His Asp Gly Ile Glu Gln Leu Asp Ser Ala Lys
                                                                     144
aac aca gat cgc ggt aca aaa tac ggt atc aac cgt aat tca gta act
                                                                     192
Asn Thr Asp Arg Gly Thr Lys Tyr Gly Ile Asn Arg Asn Ser Val Thr
tac ggc gta ttc ggc ggt tac caa att tta aac caa gac aaa tta ggt
Tyr Gly Val Phe Gly Gly Tyr Gln Ile Leu Asn Gln Asp Lys Leu Gly
tta gcg gct gaa tta ggt tat gac tat ttc ggt cgt gtg cgc ggt tct
                                                                     288
Leu Ala Ala Glu Leu Gly Tyr Asp Tyr Phe Gly Arg Val Arg Gly Ser
gaa aaa cca aac ggt aaa gcg gac aag aaa act ttc cgt cac gct gca
Glu Lys Pro Asn Gly Lys Ala Asp Lys Lys Thr Phe Arg His Ala Ala
cac ggt gcg aca atc gca tta aaa cct agc tac gaa gta tta cct gac
His Gly Ala Thr Ile Ala Leu Lys Pro Ser Tyr Glu Val Leu Pro Asp
tta gac gtt tac ggt aaa gta ggt atc gca tta gta aac aat aca tat
                                                                     432
Leu Asp Val Tyr Gly Lys Val Gly Ile Ala Leu Val Asn Asn Thr Tyr
aaa aca ttc aat gca gca caa gag aaa gtg aaa act cgt cgt ttc caa
Lys Thr Phe Asn Ala Ala Gln Glu Lys Val Lys Thr Arg Arg Phe Gln
145
agt tot tta att tta ggt gcg ggt gtt gag tac gca att ctt cct gaa
Ser Ser Leu Ile Leu Gly Ala Gly Val Glu Tyr Ala Ile Leu Pro Glu
                 165
tta gcg gca cgt gtt gaa tac caa tgg tta aac aac gca ggt aaa gca
                                                                     576
Leu Ala Ala Arg Val Glu Tyr Gln Trp Leu Asn Asn Ala Gly Lys Ala
age tac tet act tta aat egt atg ggt gea act gae tac egt teg gat
Ser Tyr Ser Thr Leu Asn Arg Met Gly Ala Thr Asp Tyr Arg Ser Asp
        195
                             200
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	ccg Pro															720
	gac Asp															768
	aca Thr															816
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gaa Glu	gct Ala 290	tca Ser	aac Asn	tta Leu	aaa Lys	ctt Leu 295	tca Ser	caa Gln	cgt Arg	cgt Arg	gcg Ala 300	gaa Glu	aca Thr	gta Val	gct Ala	912
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	tac Tyr															1008
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243

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576

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280

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Val Ile Gly Thr Asn Glu Leu Lys Val Asp Glu Lys Arg Val Glu Glu
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Ala His Tyr Ala Lys Asn Arg Gln Leu Thr Glu Asn Ile Arg Asn Val
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Ile Leu Glu Asn Pro Arg Leu His Gln Val Leu Asp Val Ile Ile Asn
Ile Gly Arg Ser Val Pro Phe Ile Ile Leu Leu Val Val Leu Leu Pro
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Val Pro Leu Ser Val Ser Ala Ile Pro Phe Phe Ala Arg Leu Thr Ser
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Ser Leu Pro Ile Leu Ile Asn Gly Ile Thr Leu Thr Leu Val Ala Leu
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aac gaa cac cct aat ttt ccg gct cgt tct tgc gta gaa gtg gct cgt
Asn Glu His Pro Asn Phe Pro Ala Arg Ser Cys Val Glu Val Ala Arg
                                                                          336
             100
                                    105
tta cca aaa gat gtt ggc att gag atc gaa gcg att gca gta cgc cga
                                                                          384
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ta
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Asn Glu His Pro Asn Phe Pro Ala Arg Ser Cys Val Glu Val Ala Arg
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277

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(43) International Publication Date 26 September 2002 (26,09,2002)

PCT

(10) International Publication Number WO 02/075507 A3

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- (22) International Filing Date: 17 January 2002 (17.01.2002)
- (25) Filing Language:
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- 15 March 2001 (15.03.2001) US
- (71) Applicant (for all designated States except US): PHAR-MACIA & UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49007 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): LOWERY, David, E. [US/US]; 1207 Woodland Drive, Portage, MI 49024 (US). FULLER, Troy, E. [US/US]; 111 Dreamfield Drive, Battle Creek, MI 49014 (US). KENNEDY, Michael, J. [US/US]; 2364 Quincy Avenue, Portage, MI 49024 (US).
- (74) Agent: WILLIAMS, Joseph, A., Jr.; Marshall, Gerstein & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).

- C12N 1/20, (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID. IL. IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG. SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
 - (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GO, GW, ML, MR, NE, SN, TD, TG).

Published:

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- (88) Date of publication of the international search report: 12 September 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Variable anti-bacterial vaccine compositions

(57) Abstract: Gram negative bacterial virulence genes are identified, thereby allowing the identification of anti-bacterial agents that target these virulence genes and their products, and the provision of gram negative bacterial mutants useful in vaccines.





Relevant to claim No.

1-41

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 A61K39/102 A61K35/74 C12N15/31 C12N15/63 CO7K14/285 C07K16/12 C12Q1/18 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Х

Minimum documentation searched (classification system followed by classification symbols) I PC $\,7\,$ C12N $\,$ A61K $\,$ C07K $\,$ C12Q $\,$ G01N

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL, EPO-Internal, WPI Data, BIOSIS, MEDLINE

Category o Citation of document, with indication, where appropriate, of the relevant passages

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х	Database accession no. AE00606/ XP002224305 nucleotides 3352-4146 & DATABASE EMBL [Online] Entry AE006064, 10 February 2001 (2001-02-10) MAY B.J. ET AL.: "Pasteurella r PM70 section 31 of 204 of the of genome"	multocida	5-23,25, 28
A	the whole document & BARBARA J. MAY ET AL.: "Com genomic sequence of Pasteurella Pm70" PROCEEDINGS OF THE NATIONAL ACA	a multocida,	1-41
X Furti	her documents are listed in the continuation of box C.	Patent family members are listed	in annex.
"A" docume consid "E" earlier of filing d "L" docume which citation "O" docume other r "P" docume	ent which may throw doubts on priority claim(s) or is cited to establish the publication date of another nor other special reason (as specified) ent referring to an oral disclosure, use, exhibition or	"I" later document published after the into or priority data and not in conflict with the conflict of the project or the terrention." "I document of particular relevance; the caranct to considered noved respect to the considered noved to particular relevance; the Cocument of particular relevance; the Cocument of particular relevance; the document is contributed with one or me ments, such conflictation being obvior in the art. "It document is contributed with one or me ments, such conflictation being obvior	eary underlying the slained invention the considered to current is taken alone slained invention ventive step when the re other such docu- us to a person skilled
Date of the	actual completion of the international search	Date of mailing of the international sea	rch report
1:	2 May 2003	16.0	5. 2003
Name and n	nalling address of the ISA European Patent Office, P.B. 5818 Patentiaan 2 NL - 2280 HV Fijswijk, Tel. (431-70) 340-2040, Tx. 31 851 epo nl, Fax: (431-70) 340-3016	Authorized officer Montero Lopez, B	
Form PCT/ISA/2	210 (second sheet) (July 1992)		





C/Continu	ation) DOCUMENTS CONSIDERED TO BE RELEVANT	PC1/03 0	2/019/1
Category *	Citation of document, with indication, where appropriate, of the relevant passages		
oulogory	Ontain of decument, with indication, where appropriate, of the relevant passages		Relevant to claim No.
	SCIENCES OF USA, vol. 98, no. 6, 13 March 2091 (2001-03-13), pages 3460-3465, XP002202785 WASHINGTON USA, page 3463, right-hand column, paragraph 2 -page 3464, left-hand column, paragraph 1		
	COONEY ET AL: "Three contiguous lipoprotein genes in Pasteurella haemolytica AL which are homologous to a lipoprotein gene in Haemophilus influenza Type b" INFECTION AND IMMUNITY, AMERICAN SOCIETY		5-23,25, 28
	OF MICROBIOLOGY, MASHINGTON, DC, US, vol. 61, no. 11, November 1993 (1993-11), pages 4682-4688, XP002148894 ISSN: 0019-9567 abstract page 4683, left-hand column, last paragraph -page 4685, left-hand column, paragraph 1; figures 3,4		
	page 4686, right-hand column, paragraph 2		
	TROY E. FULLER ET AL.: "Identification of Pasteurella multocida virulence genes in a septicemic mouse model using signature-tagged mutagenesis" MICROBIAL PATHOGENESIS, vol. 29, 2000, pages 25-38, XP002224304 the whole document		1-41
		ļ	

orm PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT



Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)		
This Inte	rmational Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:		
1.	Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:		
2.	Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:		
	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).		
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)		
This Inter	national Searching Authority found multiple inventions in this international application, as follows:		
	see additional sheet		
1. 🔲 🛔	As all required additional search fees were timely paid by the applicant, this International Search Report covers all earchable claims.		
2	is all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment any additional fee.		
0	as only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.: 1-41 partially		
4. N	o required additional search fees were timely paid by the applicant. Consequently, this International Search Report is stricted to the invention first mentioned in the claims; it is covered by claims Nos.:		
Remark or	The additional search fees were accompanied by the applicant's protest. X No protest accompanied the payment of additional search fees,		

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-41 partially

Gram-negative bacteria comprising a mutation in a gene of sequence SEQ ID NO:1 resulting in decreased activity of the gene product; immunogenic composition comprising the bacteria; method of producing such mutant bacteria; nucleotide sequence comprising SEQ ID NO:1, vector and host cell comprising the same and use thereof to produce a polypeptide; encoded polypeptide of sequence SEQ ID NO:2; antibody against it; use of the polypeptide of sequence SEQ ID NO:2 for identifying antibacterial agents.

2. Claims: 1-41 partially

Idem as subject 1 for, respectively sequences SEQ ID NO:3 and 4; 7 and 8; 9 and 10; 21 and 22; 25 and 26.

3. Claims: 1-4, 21-23, 27, 28 partially

Gram-negative bacteria comprising a mutation in a gene of sequence SEQ ID NO:27 resulting in decreased activity of the gene product; immunogenic composition comprising the bacteria; nucleotide sequence comprising SEQ ID N

Claims: 1-41 partially

Idem as subject 1 for, respectively, sequences SEQ ID NOs:29 and 30; 39 and 40; 41 and 42; 51 and 52; 53 and 54; 55 and 56.

5. Claims: 1-28 partially

Gram-negative bacteria comprising a mutation in a gene of sequence SEQ ID NO:57 resulting in decreased activity of the gene product; immunogenic composition comprising the bacteria; method of producing such mutant bacteria; nucleotide sequence comprising SEQ ID NO:57.

6. Claims: 1-41 partially

Idem as subject 1 for, respectively sequences SEQ ID NOs:58 and 59; 60 and 61; 68 and 69; 72 and 73; 74 and 75; 76 and 77; 78 and 79; 80 and 81; 82 and 83; 84 and 85; 104 and 105; 108 and 109; 112 and 113; 116 and 117; 118 and 119; 120 and 121; 122 and 123; 124 and 125; 126 and 127; 128 and 129; 130 and 131

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claims: 5-26, 29-41 partially

Attenuated Pasteurellaceae bacteria comprising a mutation in a gene of sequence SEQ ID NO:11; immunogenic composition containing it; method of producing such mutant bacteria; nucleotide sequence comprising SEQ ID NO:11, vector and host cell comprising the same and use thereof to produce a polypeptide; encoded polypeptide of sequence SEQ ID NO:12; antibody against it; use of the polypeptide of sequence SEQ ID NO:22 for identifying antibacterial agents.

8. Claims: 5-26, 28-41 partially

Idem as subject 36 for, respectively, sequences SEQ ID NOs:13 and 14; 15 and 16; 17 and 18; 19 and 20; 23 and 24; 31 and 32; 33 and 34; 35 and 36; 37 and 38; 70 and 71; 100 and 101; 102 and 103; 106 and 107; 110 and 111; 114 and 115; 132 and 133; 134 and 135; 136 and 137; 138 and 139; 140 and 141; 142 and 143; 144 and 145; 146 and 147; 148 and 149; 150 and 151; 152 and 153; 154 and 155; 156 and 157; 158 and 159; 160 and 161

9. Claims: 5-26 partially

Attenuated Pasteurellaceae bacteria comprising a mutation in, respectively a gene of sequence SEQ ID NO:162 and 163; immunogenic composition containing it; method of producing such mutant bacteria; nucleotide sequence comprising SEQ ID NO:162 or 163.

10. Claims: 5-26, 28-41 partially

Idem as subject 36 for, respectively, sequences SEQ ID NOs:164 and 165; 166 and 167; 168 and 169; 170 and 171; 172 and 173; 174 and 175

name 2 of 2

(19) World Intellectual Property Organization International Bureau





(43) International Publication Date 26 September 2002 (26,09,2002)

PCT

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- (25) Filing Language:

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English

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 - 15 March 2001 (15.03.2001) US
- (71) Applicant (for all designated States except US): PHAR-MACIA & UPJOHN COMPANY [US/US]; 301 Henricita Street, Kalamazoo, MI 49007 (US).
- (72) Inventors: and
- (75) Inventors/Applicants (for US only): LOWERY, David, E. [US/US]; 1207 Woodland Drive, Portage, MI 49024 (US). FULLER, Trov, E. [US/US]; 111 Dreamfield Drive,
- Battle Creek, M1 *****

 (US/US); 2364 Quincy Avenue, Portage, M1 ****

 (74) Agent: WILLAM, Joseph, A., Jr.: Marshall, Gerstein & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, II. 60606 (US).

- (81) Designated States (national): AE, AG, AL, AM, AT. AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM. HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN. YU. ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GII, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FL FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BE, BJ, CE, CG, CI, CM, GA, GN, GO, GW, ML, MR, NE, SN, TD, TG).

Published:

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 - with amended claims
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Date of publication of the amended claims: 11 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

02/075507 A3

AMENDED CLAIMS

[received by the International Bureau on 11 July 2003 (11.07.03) original claims 1 to 41 have been amended by claims 1 to 29

WHAT IS CLAIMED IS:

- An attenuated Mannheimia bacteria comprising a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOS: 166, 168, 170, 172 and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.
 - The Mannheimia bacteria of claim 1 wherein said mutation results in decreased expression of a gene product encoded by the mutated gene.
- 10 3. The Mannheimia bacteria of claim 1 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
 - The Mannheimia bacteria of claim 1 wherein said mutation results in deletion of all or part of said gene.
 - The Mannheimia bacteria of claim 1 wherein the Mannheimia bacteria is Mannheimia haemolytica.
- 6. The *Mannheimia* bacteria of claim 5 wherein said mutation results in
 20 decreased expression of a gene product encoded by the mutated gene.
 - 7. The *Mannheimia* bacteria of claim 5 wherein said mutation results in expression of an inactive gene product encoded by the mutated gene.
- The Mannheimia bacteria of claim 5 wherein said mutation results in deletion of all or part of said gene.
 - $9. \qquad \text{An immunogenic composition comprising the bacteria according to} \\$ any one of claims 1 through 8.

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 A vaccine composition comprising the immunogenic composition according to claim 9 and a pharmaceutically acceptable carrier.

- The vaccine composition according to claim 10 further comprising an
 adjuvant.
 - 12. A method for producing a gram-negative bacteria mutant comprising the step of introducing a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOS: 166, 168, 170, 172, and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.

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- 13. A method for producing an attenuated Mannheimia bacteria comprising the step of introducing a mutation in a gene represented by a nucleotide sequence set forth in any one of SEQ ID NOS: 166, 168, 170, 172, and 174 or a species homolog thereof, said mutation resulting in decreased activity of a gene product encoded by the mutated gene.
- A purified and isolated Mannheimia polymucleotide comprising a
 nucleotide sequence selected from the group consisting of nucleotide sequences set forth in SEO ID NOS: 166, 168, 170, 172 and 174.
 - A purified and isolated Mannheimia polynucleotide comprising a nucleotide sequence as set forth in SEQ ID NO: 166.
 - 16. A purified and isolated polynucleotide encoding a Mannheimia virulence gene product, or species homolog thereof, selected from the group consisting of:
 - a) the polynucleotide according to claim 14;

 b) polynucleotides encoding a polypeptide encoded by the polynucleotide of (a); and

c) polynucleotides that hybridize to the complement of the polynucleotides of (a) or (b) under moderate stringency conditions.

A purified and isolated Mannheimia polynucleotide encoding a
polypeptide selected from the group consisting of polypeptides having amino acid
sequences set forth in SEQ ID NOS: 167, 169, 171, 173, and 175.

10 18. The polynucleotide of claim 17 which is a DNA.

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- 19. A vector comprising the DNA of claim 18.
- The vector of claim 19 that is an expression vector, wherein the DNA
 is operatively linked to an expression control DNA sequence.
 - A host cell stably transformed or transfected with the DNA of claim 18 in a manner allowing the expression of the encoded polypeptide in said host cell.
- 20 22. A method for producing a recombinant polypeptide comprising culturing the host cell of claim 21 in a nutrient medium and isolating the encoded polypeptide from said host cell or said nutrient medium.
 - 23. A purified polypeptide produced by the method of claim 22.

 A purified polypeptide comprising a polypeptide selected from the group consisting of polypeptides having amino acid sequences set forth in SEQ ID NOS: 167, 169, 171, 173, and 175.

An antibody that is specifically reactive with the polypeptide of claim

26. The antibody of claim 25 that is a monoclonal antibody.

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27. A method of using the monoclonal antibody of claim 26 for identifying a bacteria of claims 1 or 5, comprising the steps of contacting an extract of bacteria with said monoclonal antibody and detecting the absence of binding of said monoclonal antibody.

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28. A method of identifying an anti-bacterial agent comprising the steps of assaying potential agents for the ability to interfere with expression or activity of gene products represented by the amino acid sequences set forth in any one of SEQ ID NOS: 167, 169, 171, 173, and 175 and identifying an agent that interferes with expression or activity of said gene products.

 A method of identifying an anti-bacterial agent comprising the steps of:

a) measuring expression or activity of a gene product as set out in
 any one of SEO ID NOS: 167, 169, 171, 173, and 175;

- b) contacting the gene product in (a) with a test compound;
- c) measuring expression or activity of the gene product in the presence of the test compound; and
- d) identifying the test compound as an antibacterial agent when
 25 expression or activity of the gene product is decreased in the presence of the test compound as compared to expression or activity in the absence of the test compound.